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A SYSTEM OF BACTERIOLOGY IN RELATION TO MEDICINE

VOLUME III

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For this Volume Dr. J. A. Arkwright, F.R.S., Dr. A. Eastwood, Professor A. Harden, F.R.S., and Dr. W. M. Scott have assisted in the editorship with regard to particular sections.

A
SYSTEM OF BACTERIOLOGY
IN RELATION TO MEDICINE

VOLUME III

BY

W. BULLOCH, P. FILDES, A. T. GLENNY, H. HENRY,
R. T. HEWLETT, R. A. O'BRIEN, S. G. PAINE, G. F. PETRIE,
MURIEL ROBERTSON, R. St. JOHN-BROOKS, W. G. SAVAGE,
A. C. THAYSEN, H. G. THORNTON, R. L. VOLLUM.

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CHAPTER I. THE ECONOMIC ASPECTS OF BACTERIOLOGY.

By A. C. Thaysen
(Royal Naval Cordite Factory, Holton Heath).

Historically the economic aspects of bacteriology claim attention, as it was the realization of the participation of micro-organisms in the production of vinegar, in the manufacture of wine and in the brewing of beer, which led to the development of the science.

There are other and more significant reasons, however, why the subject of economic bacteriology should not be lost sight of. To an increasing extent it is being realized that the unique catalytic properties of bacterial enzymes may become invaluable assets to many industries not necessarily connected with those commonly described as the fermentation industries. And in equal measure it is being understood that bacterial activity may involve industries in serious pecuniary losses through the deterioration of their raw materials, or through interference with the normal course of manufacturing processes.

It is in the light of both of these possibilities, in the utilization and in the prevention of bacterial activity, that the economic aspects of bacteriology will be dealt with in these pages. But it should be emphasized that specialized subjects, such as dairy bacteriology, soil bacteriology, sewage bacteriology and canning bacteriology are not included here. The problems connected with brewing, wine production and vinegar-making have been entirely omitted, as these subjects, in the course of time, have become too highly specialized to be dealt with in the available space in even a quite superficial manner. Readers interested in the art of brewing and its allied subjects are therefore referred to the standard textbooks on this subject.

A separate section has been reserved for the discussion of the formation of products of economic importance, such as peat, coal, shale oil and natural gas, the formation of which is now considered by some to be the result of bacterial activity.

In closing these introductory remarks it is felt that attention should be drawn to the use which has been made of the term ‘ bacteriology ’ in these pages. It here embraces knowledge not only of the true schizomycetes, but of the actinomycetes and of many lower fungi.

The Application and Prevention of Bacterial Activities in Industry.

BAKING.

The proper regulation of the fermentation during dough-raising is perhaps the most outstanding problem in bread-making, since the rate of fermentation is dependent on a great many variables, such as the flour itself, the admixtures to the flour, and the natural microflora of the flour (Brewster-Morison, 1923). The purpose of the fermentation is to ensure a uniform dispersion of gas in the dough. It must be conducted in such a way that the physical properties of the dough remain undamaged. A prevention of the destruction of the gluten of the flour is of the greatest importance, since the gas-retaining properties of the dough is conditioned by this constituent of the flour.

Most, if not all, flours contain amongst their natural microflora species which are capable of producing appreciable quantities of gas—carbon dioxide and hydrogen. Holliger (1902), who undertook an exhaustive investigation of the dough fermentation, refers to two species—*B. levans* and another short, *coli*-like rod, forming yellow colonies on agar. It is highly probable that the first types of leaven used by man consisted essentially of such bacteria, admixed no doubt with many other types derived from the flour, notably species of lactic acid bacteria. Such leavens are still in use in countries where rye bread is consumed. An analysis of the microflora present in rye dough was carried out by Budinoff (1903). He isolated in one case no less than ten species, of which two were yeast and the remainder bacteria. Of the bacteria two species were lactic acid bacteria, one probably an acetic acid bacterium, and apart from occasional cocci, the remainder were spore-bearing rods of the type of *B. mesentericus*. Budinoff attributes the gas evolution to the yeast species present, the only types found which were capable of fermenting carbohydrates with marked production of gas.

The introduction of yeast into dough for improving its baking qualities is a custom which, in disguise, can be traced to the bakers of the Roman Empire. It was known to them that an addition of young wine to dough greatly improved the appearance and texture of the resulting bread. Instead of wine, different types of barm were subsequently used, liquids prepared in the bakehouse from flour and water, with or without malt, and inoculated with a sample of well-raised dough. The preparation of these barms was a matter of considerable skill, and countless are the numbers of recipes which from time to time have been recommended for this purpose. This is not surprising seeing that the microflora of the barm had to suppress the putrifying and the acid-producing bacteria of the flour and secure the evolution of gas in the dough at a rapid and uniform rate. In the complete absence of bacteriological knowledge, the production of such a barm must have been a highly complicated task.

Barms of various descriptions were in general use until the eighteenth century, when they became replaced by pressed yeast. To-day pressed yeast—baker's yeast—is almost exclusively used and has considerably simplified and safeguarded the preliminary stages of bread-making. By the use of pressed yeast as a leaven the introduction of many undesirable bacteria into the dough is prevented, and the activity of the normal microflora of the flour can be kept under control, since the time taken for the dough to rise—eight to ten hours—is too short to allow of any appreciable development.

But in spite of the simplification of dough fermentation, achieved through the introduction of baker's yeast as a leaven, the rising of the dough remains a complex phenomenon and must involve other reactions than the production of carbon dioxide and ethyl alcohol, the fermentation products of the added yeast. In most bakeries sugar is not added to the flour on working it up into dough. Nor is such addition necessary, since wheat flour contains between 2 and 3 per cent. of fermentable carbohydrates (Jago, 1895), sufficient to produce the gas required for the raising of the dough.

In addition to this sugar, yeast-fermentable carbohydrates are produced, probably from the starch, during the raising of the dough through the activity of amylolytic enzymes contained in the flour itself. It is unlikely that the hydrolysis of the starch can be the result of the activity of the normal microflora of the flour. The short time allowed for the rising of the dough would be quite insufficient to allow of that.

It has not been definitely established to what extent the activity of the yeast influences the protein constituents of the flour. That an action takes place is certain, and is traceable in the gas-retaining and coagulating properties of the gluten, properties which become adversely affected if the dough is allowed to rise for too long.

In reviewing the subject of dough fermentation reference must be made to the fate of the microflora of the dough during baking, and to the activity of bacteria in the finished bread. Though the baking is usually carried out between the temperatures of 230 to 248° C., this exposure is not of sufficient duration to raise the temperature in the interior of a loaf of the usual size to beyond 101 to 103° C., a temperature which is insufficient to destroy the spores of many bacteria, particularly when the reaction of the dough has approached closer to the neutral point than desirable, i.e. has shown a pH value greater than 5·0 (Jessen-Hansen, 1911).

In such cases 'ropy bread' may result, particularly when subsequent storage of the bread during cooling, and before consumption, takes place in a damp atmosphere. The responsible organism, *B. mesentericus panis viscosi*, was first isolated by Vogel (1897) and subsequently studied in detail by Thomann (1900) and others, and shown to be a frequent inhabitant of flour, its spores, as already mentioned, surviving the baking process when the pH value of the dough is increased beyond 5·0.

B. mesentericus panis viscosi is closely related morphologically and physiologically to *B. mesentericus*.

Since the introduction of 'baker's yeast' for the raising of dough, the bacteriological problems facing the baker have been greatly simplified, and are limited almost entirely to ropiness and to mildewing, which may occur on storage. The latter fault is considered by Welte (1895) to be due to an infection of the crust with lower fungi subsequent to baking. Both types of damage can be very largely avoided by the introduction of general hygienic principles in the bakehouse, and ropiness in particular by the adjustment of the hydrogen ion concentration of the dough to that most suitable for the development of the yeast (pH value 5·0).

THE BACTERIAL DECOMPOSITION OF PECTIN.

Retting.

The retting of fibre plants, particularly of flax, is one of the most ancient applications of bacteria to industry, more ancient perhaps than the manufacture of alcoholic beverages and of the preparation of leaven for making dough. Definite evidence is available showing that the Swiss lake-dwellers were familiar with the art of the retting of flax. In countries like Egypt highly developed retting methods were in use even during the earliest dynastic times. While the Swiss lake-dwellers adopted a retting process dependent on stagnant water, the Egyptian process was based on the utilisation of the slow-flowing waters of the river Nile. Both methods are still extensively used, the former in Ireland and Italy, the latter in Holland, Belgium and Germany.

In both cases the principles are the same and comprise a physical, a biological, and a mechanical stage. In this connection it may be desirable to recall that the flax stem consists essentially of a central cylinder of wood, on the outside of which are found bundles of thick-walled fibres, the flax fibres, embedded in a matrix of parenchymatous tissue. The individual fibres of the bundles are held together by a substance which Störmer (1904) regards as lignified pectin. It is less readily attacked by bacteria than the ordinary pectin by which the fibre bundles are joined to the surrounding parenchymatous tissue. On the outside the flax stem is covered by an epidermis, the cuticle, which possesses pores—stomata—through which bacteria can penetrate to the parenchymatous tissue.

During the physical stage of retting the fibre plants are submerged in water, which, among other functions, extracts from the tissues of the plants a number of substances such as carbohydrates, glucosides, tannin, nitrogen compounds and colouring matter, and gradually expels the air entangled in and around the tissues. The extraction of these various soluble substances by the surrounding water renders possible a vigorous development of many of the bacteria normally present in the water and on the fibre plants. During this development two phases are discernible. During the first, the preliminary biological phase, a great variety of aerobic and facultative anaerobic bacteria develops at the expense of the substances extracted from the plant tissues. By this development the available oxygen is utilized and anaerobic conditions established. An opportunity is thus given, during the second or principal biological phase, for the typical anaerobic retting bacteria to develop. These bacteria are almost invariably present in agricultural soils and, in consequence, are found also on the textile plants. They attack and destroy the pectin composing the middle lamella of the parenchymatous tissues of the plants undergoing retting, and thereby separate the fibre bundles from the cortex and the woody core. The completion of the destruction of the middle lamella renders it possible to remove the fibre bundles more or less free from other tissues. This is done during the mechanical stage.

Though bacteria play a comparatively unimportant role during the physical stage of retting, this phase is nevertheless of great importance from a biological point of view, since the microflora developing subsequently will depend both on the nature of the substances extracted from the fibre plants and on the concentration of these substances. Further, the entry of water into submerged tissues not only expels the air present, but swells the tissues sufficiently to burst the cortex. Orifices are thereby formed through which the pectin-decomposing bacteria can enter the tissues (Müller and Tobler, 1922). The swelling of the tissues also favours the removal into the surrounding liquid of the decomposition products formed during the retting, and thereby assists in ensuring a uniform loosening of the fibres.

Very early during the preliminary biological stage, sometimes within 24 hours of the immersion of the tissues, the surrounding water becomes cloudy through the development of a secondary flora. A detailed study of this microflora has not yet been made. It is known, however (Hauman, 1902), that *B. mesentericus*, *B. subtilis*, *B. mycoides*, *Oidium lactis*, *B. coli*, *Cladosporium herbarum*, as well as various *Torula* species, lactic acid-producing bacteria, and denitrifying bacteria form part of it. It is questionable whether the *B. coli* met with is identical with *B. coli communis* Escherich. More probably it belongs to the group of *coli*-like bacteria, which is frequently met with in soil, water, and on vegetation. Hauman's (1902) statement that he observed two different types of *B. coli* supports this point of view.

It has been reported by Hauman (1902), Behrens (1903), and Beijerinck and van Delden (1904) that several members of the secondary microflora are capable of decomposing pectin. They might therefore be assumed to take part in the actual retting. However, Eyre and Nodder's (1924) observation definitely disposes of this possibility, since these writers found that only a small proportion of the available pectin is decomposed during the preliminary biological stage. Where the retting is carried out in stagnant water the secondary microflora gradually forms a thick white or greyish coating on the surface of the liquid. This layer has been shown to contain the mycelium of various fungi as well as the cells of a number of spore-forming and non-spore-forming bacteria. It contains also numerous entangled gas bubbles containing a mixture of hydrogen, carbon dioxide and nitrogen.

At ordinary autumn temperatures the preliminary biological stage is completed in about 100 hours. Before it is completed the anaerobic pectin-decomposing bacteria have begun their attack on the middle lamella.

These bacteria which in the course of time have been assigned various names, such as *Granulobacter pectinovorum* and *Clostridium pectinovorum*, were studied in detail under the name of *B. amylobacter* by Bredemann (1909). More recently they have been investigated by Donker (1926), who devoted special attention to the fermentation products of the types examined by him. Apart from decomposing pectin they ferment carbohydrates with the production of acetic and butyric acids, acetone and butyl alcohol, and with the evolution of hydrogen and carbon dioxide. They are capable also of fixing atmospheric nitrogen. They produce oval spores of a diameter greater than that of the cell. On sporulation, therefore, the cell frequently appears spindle-shaped. Characteristic for most strains is the accumulation within the cell at certain stages of their life cycle of appreciable quantities of a substance, granulose, which stains dark purple with iodine.

Donker groups all of these anaerobic spore-bearing bacteria in a genus *Clostridium*, and shows that among them two fairly clearly defined sub-groups may be discerned. In the one the organism is unable to hydrogenate the organic acids produced as intermediate fermentation products from carbohydrates to their corresponding neutral derivatives, butyl alcohol, ethyl alcohol and acetone. To this group belong the true butyric acid bacteria, *B. saccharobutyricus* for instance. In the other are ranged what broadly speaking may be termed the butyl alcohol bacteria. Here, when the reaction of the surrounding medium is favourable, the primarily produced butyric acid and acetic acid are reduced to butyl alcohol, ethyl alcohol or acetone. In the case of some of the butyl alcohol bacteria, *Clostridium pectinovorum*, for instance, and *Clostridium pasteurianum*, it is essential to add calcium carbonate to the medium to secure this favourable reaction; in the case of others, *Clostridium butylicum*, such addition is unnecessary, and may in fact be undesirable. The type originally described by Beijerinck and van Delden (1904) as the retting bacterium was *Clostridium* (*Granulobacter*) *pectinovorum*.

A microscopic examination during the principal biological stages of the plant tissues undergoing retting shows large numbers of the cells of *Clostridium pectinovorum* accumulated between the fibre bundles and the cortex. They are often embedded in mucus. The destruction of the pectin gives rise to the evolution of hydrogen and carbon dioxide, and to the formation of butyric and acetic acids. These products, as well as protein decomposition products formed partly by the retting bacillus and partly by members of the secondary microflora, are responsible for the objectionable odours emanating from retting pits.

During the last stages of the principal biological phase the activity of the retting bacillus may extend to the lignified pectin (Stőrmer, 1904), which cements the individual fibres of the fibre bundles. Where this is the case over-retting occurs, a most serious fault in retting. There are several methods in existence for determining when the retting has reached its optimum. Most of these methods require experienced judgment. Under ordinary autumn conditions, and in stagnant water, the principal biological stage requires from 80 to 100 hours to be completed.

It is commonly observed that the gas evolved during retting contains a percentage of methane. This, as well as some of the hydrogen and carbon dioxide, is produced by cellulose-destroying bacteria of Omelianski's (1895) *B. methanigenes* type, introduced into the retting liquors with soil and plant debris. Where these bacteria occur in large numbers, serious dangers exist of contaminating and possibly of destroying the fibres.

The question of the time required for the completion of the retting process was of little importance so long as the retting of flax remained a cottage industry. In fact, in certain parts of Belgium the retting was deliberately interrupted when half completed, and was restarted after a drying of the fibre plants. This was done to avoid the danger of over-retting. With the centralization of the industry, however, a rapid completion of the retting process became essential. Various methods have been suggested to achieve this. The first, and the most obvious, was proposed by an American technologist Schenck (Hodges, 1854), and consisted in conducting the retting at an increased temperature (37° C.). The dangers of over-retting were seriously increased by this treatment, however, and many factories using the warm water retting process, therefore, prefer to work at a temperature of 28 to 29° C.

Another method of increasing the rate of retting and of securing a more homogeneous product consists in adding pure cultures of pectin-decomposing bacteria to the retting water. The earlier attempts were not uniformly successful and opinions are still divided as to the value of this method. Carbone (1922) claims considerable success when using a special type of pectin-fermenter, *B. felsineus*, a spore-forming rod isolated from Italian retting pits.

Apart from the efforts made to increase the speed of the retting, considerable attention has been given to the study of means for avoiding the unpleasant odours associated with retting processes as carried out in stagnant water. The most successful means by which this is achieved appears to be the introduction of oxygen into the retting water. This is the case in Rossi's aerobic tank retting for instance, where pure cultures of another pectin-decomposing bacterium, *B. comesii*, is added to the retting liquor. The ret is conducted at 28 to 30° C., and air, at the rate of 200 litres per minute, is blown through the tank, which contains 50,000 litres of retting liquor. A still more effective method of semi-aerobic retting is that recommended by Ochmann (1922), in which the retting liquor is continuously renewed and replaced by fresh water, aerated through spraying.

Aerobic retting is achieved also in 'dew-retting', a method which has been known for centuries and which is still carried out extensively in Russia and in the Baltic States. Here the fibre plants are spread out in thin layers on the ground, during summer, early spring or winter. The ground selected should preferably be heathland and should contain a minimum of bacteria. The presence of low shrubs is an advantage, preventing the fibre plants from coming into contact with the soil itself.

Hauman (1902), who studied samples of dew-retted flax, found a great variety of micro-organisms, including *B. coli cummunis*, *B. mesentericus*, *B. subtilis*, *B. mycoides*, *B. termo*, *B. fluorescens liq.*, *M. roseus*, *Penicillium glaucum*, *Mucor mucedo*, and *Cladosporium herbarum*. Of these he regarded the last named as the causative agent. This was contradicted by Behrens (1902), however, but has more recently been confirmed by Ruschmann (1923).

THE BACTERIAL DECOMPOSITION OF CELLULOSE.

Preparation of Farmyard Manure.

Another economic application of bacteria, the preparation of farmyard manure, was an important agricultural practice centuries before the existence of bacteria had been realized. The conditions governing the production of farmyard manure were common knowledge long before Reiset (1856) drew attention to this interesting problem, and gave an impetus to researches which were to throw light on the important processes involved in the natural elimination of vegetable debris and in the formation of peat and coal.

It is not possible to deal with these investigations in the space available. They have been discussed recently at some length by Thaysen and Bunker (1927), and a reference to this source of information must suffice. It must be recorded, however, that a successful method has been worked out during recent years for the preparation of synthetic farmyard manure, a product possessing all the valuable agricultural properties of natural farmyard manure, without having been mixed with the fæcal matter of the animals of the farmyard. This method, the outcome of the researches of Hutchinson and Richards (1921), will undoubtedly become of the greatest value in many countries where surplus straw and other vegetable refuse is now being disposed of by the wasteful method of burning.

Reference must be made also to the attempts, so far sporadic, to utilize on a technical scale, and under complete control, the activity of those bacteria of the manure heap which are responsible for the evolution of methane during the ripening of the heap. A plant is stated by Richards to have been worked by the present Lord Iveagh at Pyrford court, in which it was possible to produce combustible gases in desired quantities by the fermentation of straw. Work on similar lines has been carried out by Fowler and Joshi (1920), by Nobel's explosives factory at Ardeer (Nathan, 1923), and by Amoore (1927) at the Rothamsted Experimental Station.

The application of synthetic farmyard manure as a binder for coal-dust briquettes has been advocated by Goodwin and White (1925). They add 4 per cent. of the manure, mixed with sodium carbonate, to coal dust, and subject the resulting paste to pressing and baking. The humus of the manure thereby becomes impervious to water, without losing its adhesive properties, and the production of a firm briquette is claimed to result.

Intestinal Decomposition of Cellulose.

The study of the intestinal decomposition of cellulose is linked with the investigation of the bacteriology of the manure heap and with van Tieghem's (1879) re-investigation of Mitscherlich's (1850) and Trécul's (1865) bacterium *Amylobacter*. For this reason the subject of cellulose digestion is dealt with at this stage.

Long before the essential part played by bacteria in digestion had been experimentally proved by Schottelius (1899, 1902, 1908), Tappeiner (1882) had associated the intestinal decomposition of cellulose with the activity of bacteria, and had come to the conclusion that it might take place either under alkaline conditions, when hydrogen and carbon dioxide would be evolved and fatty acids produced, or under acid conditions when, in addition to fatty acids formed, the gas given off would consist of a mixture of methane and carbon dioxide. In both cases the breakdown of the cellulose would occur on lines similar to those which had already been shown to be characteristic for the manure heap.

Tappeiner (1882) ascribed the nutritive value of cellulose to the fatty acids produced from it during digestion, and thus allocated to this carbohydrate a much lower feeding value than to starch or sugar. Later, Kellner and his collaborators (1900) showed, in their feeding experiments on cattle, that the nutritive value of cellulose is much higher, and under suitable conditions may equal that of starch. The economic importance of this observation was very great. It indicated that a comparatively expensive feeding material, grain, could be replaced by a much cheaper supply, cellulose, in the form of wood, for instance, which had been treated previously to remove its lignin.

Theoretically, Kellner's observation was equally important. It proved the fallacy of Tappeiner's conception of the intermediate stages of cellulose digestion, and opened the way for the interpretation given by Pringsheim (1923) and by Khouvine (1923) that the participation of bacteria in cellulose digestion is limited to the hydrolysis of the cellulose to carbohydrates, such as cellobiose or perhaps glucose, which may be absorbed by the intestine.

While knowledge of the biochemistry of cellulose digestion has thus been materially advanced, the bacteriology of the reaction still remains largely unexplored. This is due partly to the technical difficulties encountered in the isolation of the responsible bacteria and partly to the misconception of the term 'cellulose' which has for long been allowed to exist. Until comparatively recently, the word cellulose was frequently used to describe the whole of the solid constituents of the cell walls of plant tissues, a mixture which is now known to contain true cellulose, hemicelluloses and pectin. Before referring to recent work on the isolation and study of cellulose-decomposing bacteria it will be of advantage to review the history of the subject.

The first observations on the fermentation of cellulose were recorded by Mitscherlich (1850) who reported having followed the gradual disintegration of slices of potato under the action of a 'ferment'. Somewhat later Trécul (1865) reported a similar resolution of plant tissues, and described three types of microscopic bodies which he associated with the disintegration. He termed them *Amylobacter*, *Clostridium* and *Urocephalum*. Van Tieghem (1879) repeated Trécul's work and showed that the microscopic bodies described by Trécul were stages of development in the life-cycle of a bacterium which he termed *Amylobacter*. He ascribed the disintegration of potato slices under water to the same bacterium and made it responsible also for the retting of flax, pointing out, however, that though it liberated the fibre bundles from the flax stems it did not attack the individual fibres. In spite of this, van Tieghem regarded *Amylobacter* as the cellulose-fermenting bacterium *par excellence*, and was to some extent justified in doing so, seeing that the expression 'cellulose', as already mentioned, was then used to designate the whole of the cell walls of plant tissues.

As a cellulose fermenter *Amylobacter*, and other related butyric acid bacteria such as *Clostridium polymyxa* Prażmowski (1879), remained firmly established in the literature for several decades. The properties of *Amylobacter* were confirmed by Hoppe-Seyler (1883) who, on microscopic examination of Swedish filter paper disintegrated in the laboratory in the presence of pond mud, reported its presence among the bacteria seen, and by van Senus (1890) who studied the bacterial decomposition of cotton wool.

Not until Omelianski (1895) undertook an exhaustive re-investigation of Hoppe-Seyler's study of the action of pond mud on pure resistant cellulose was doubt thrown on the functions of *Amylobacter* as a cellulose-fermenting bacterium. Omelianski in his publication was very insistent on the point that the two cellulose-fermenting bacteria isolated by him from the disintegrated filter paper used in his experiments in no way resembled *Amylobacter*. This undoubtedly was correct, and it is a fact that wherever *Amylobacter* or any other strain of butyric acid bacteria has been tested in pure culture for its action on pure cellulose, either in the form of cotton wool or filter paper, it has invariably been found incapable of attacking the carbohydrate. It can be claimed to have been definitely established that the action of all butyric acid bacteria on plant tissues is limited to the removal of starch, sugars and pectin, the resolution of the latter being the cause of the disintegration observed by Mitscherlich and Trécul.

The search for cellulose-fermenting bacteria, notably for types which could perform the conversion in the intestine under anaerobic conditions, has been delayed, as already mentioned, owing to the technical difficulties met with in getting these bacteria to develop on laboratory media. The only successful effort in this direction is that recorded by Khouvine (1923). Her observations are of particular interest in bringing forward experimental evidence in support of Pringsheim's (1923) contention that the function of the cellulose-fermenting bacteria in the intestine is that of a hydrolytic agent, converting the insoluble polysaccharide, cellulose, into assimilable carbohydrates. Of the variety of the microflora capable of performing this important hydrolytic function there is at present but the scantiest information. It has been suggested by Hopffe (1919) that an *Aspergillus* species might participate in the intestinal resolution of cellulose. But this is improbable, since the hydrolytic action of this fungus is too slow. Perhaps methods of direct microscopic examination might assist in throwing light on the problem. Such methods have been recommended by Henneberg (1922).

The Bacteriological Problems of the Textile Industries.

Practically all textile fibres, with the exception of those made from cellulose acetate, can be used as a food substance by bacteria and by many of the lower fungi. They are, therefore, exposed to attack and destruction by these micro-organisms, an attack which becomes possible, according to Fleming and Thaysen (1920), as soon as the moisture content of the fibres exceeds 9 per cent.

This action of micro-organisms on textile fibres has long been known under various trade names such as 'mildewing' or 'felting'. But the extent of its importance was revealed only in comparatively recent years when statistical enquiries in a number of countries showed that as much as 25 per cent. of many textile raw materials, for instance cotton, may be rendered useless for manufacturing purposes through the action of bacteria during their storage and transit after harvesting.

Various chemicals, such as zinc chloride, phenol and phenol derivatives, have been suggested from time to time as means of checking and preventing mildewing and felting, but for various reasons these preventives have proved inefficient. The question of the most suitable antiseptic has recently received renewed attention by Morris (1927), and a large number of likely substances have been tested by him without any being found which could be claimed to possess the many properties required by a suitable mildewing antiseptic. The fact remains, therefore, that reduction of moisture to below 9 per cent. offers the only safeguard against the development of bacteria and fungi on textile fibres and fabrics. This protection, unfortunately, can only be adopted in certain cases, notably during storage and transit. In the actual manufacture of textiles addition of moisture to the raw material is frequently necessary. Here the only available safeguard against bacterial attack is, as pointed out by Bright, Morris and Summers (1924), the introduction during manufacture of improved methods of cleanliness in the mills, which would render it more difficult for bacteria to develop and would prevent the spread of an already existing infection.

The Bacterial Problems of the Wood Industries.

In spite of the protection afforded by the presence of lignin, wood is as exposed to attack and decay by micro-organisms as textile fibres, and the storage and handling of raw and manufactured timber cannot be safely undertaken without attention being paid to hygienic principles. This is by no means universally recognized and is the reason for the very heavy pecuniary losses suffered by the wood industries, losses which, according to Canadian estimates (Acree, 1919), amount to from five to ten million dollars annually for that country alone.

The risk of contamination of the wood commences with the felling of the timber and is particularly great where the felling is done while the trees are 'in sap'. The infecting micro-organisms are usually fungi, though bacteria have been reported by Schmitz (1919) to participate in the decay through creating favourable conditions for the growth of the fungi. The active fungi, of which a large number exists, have recently been studied by Canadian and American investigators, notably by von Schrenck and his collaborators (1902 and 1909), Long and Harsch (1918), Fritz (1923), Hubert (1924), and many others. It has been shown that wood-destroying fungi find suitable conditions for growth on the forest floor and in timber yards, where bark and wood refuse are allowed to accumulate. From hence they infect the wood in course of manufacture. Their spread on wood is restricted only by moisture conditions. It has been shown by Hoxie (1920), Snell (1921), and Scheible (1923) that the moisture limits allowing the development of fungi are from 20 to 60 per cent., except in the case of dry rot, *Merulius lacrymans*, which, through special provisions, is able to spread over wood with a much lower moisture content. The application of the observation that fungi are unable to develop on wood containing more than 60 per cent. of moisture has been recommended by Snell (1921) for protective purposes by storage of wood under water.

The damage caused to wood by the various wood-destroying fungi is not limited to a loss in strength of the timber through perforation and weakening of the tissues, but involves losses and chemical changes to the lignin and to the cellulose, which render the latter unsuitable for the manufacture of paper. These changes have been studied in recent years by Rose and Lisse (1917), Johnsen and Lee (1923), and Bray and Andrews (1924), and their effect on the manufacture of paper by Kress and collaborators (1919), Sutermeister (1921), Hawkins (1923), and others. They include a discoloration of the finished paper, notably in the case of paper prepared from mechanical wood pulp, a discoloration which may be due to pigments secreted by the active fungi or to the chemical changes wrought during the decay.

This pigmentation of paper should not be mistaken for the mildewing to which books and paper are exposed when stored damp. According to Sée (1919) the fungi responsible for this deterioration are to be regarded as true paper inhabitants and are different from the many types of fungi which may develop on wood. These 'paper fungi' are able to develop on pure cellulose, in the absence of added nitrogen, and their spread under damp conditions therefore is very difficult to check.

THE BACTERIAL DECOMPOSITION OF HEMICELLULOSES.

Spontaneous Heating of Hay and Similar Reactions.

The conception is widespread that the decomposition of cellulose in plant tissues undergoing decay is the essential feature of the processes occurring in the manure heap. It is gradually being realized, however, that the destruction of the cellulose is not only anticipated by, but perhaps even dependent on, a decomposition of the hemicelluloses of these plant tissues, a decomposition which is essential from other points of view than that of establishing suitable temperatures, as suggested by Gayon (1884) and others. This subject is likely to receive increased attention in the future owing to its great theoretical and practical interest.

The decomposition of hemicelluloses by bacteria is of considerable importance in other respects. The spontaneous heating of hay, the preparation of silage and the making of sauerkraut and similar delicacies depend essentially on the bacterial decomposition of hemicelluloses.

The spontaneous heating of hay was investigated in considerable detail by Miehe (1911), who came to the conclusion that it was due primarily to the activity of two types of bacteria, *B. coli* forma *foenicola* and *B. calfactor*. *B. calfactor*, which has its optimum temperature for growth at 55° C., resembles *B. mesentericus* in its development on potatoes. Contrary to *B. mesentericus* its stab cultures show marked branching, and its spores are formed subterminally instead of centrally. According to Lehmann and Neumann (1912), *B. calfactor* may be isolated from almost any sample of fermenting hay. *B. coli* forma *foenicola* is claimed by Miehe to be responsible for the initial heating of the hay, which brings the temperature of the hayrick to 40° C., a temperature at which the activity of *B. calfactor* becomes marked, thereby raising the temperature of the hay to between 70° C. and 75° C. Temperatures above these are not due to the activity of bacteria, as shown both by Miehe and by Haldane and Makgill (1923), but to the rapid absorption of oxygen by the hay after its prolonged heating at the lower temperatures, and its consequent drying.

Several investigators deny that bacteria perform any essential function in the spontaneous heating of hay and claim the phenomenon to be due to chemical changes (Boekhout and de Vries, 1904), or to the functioning of oxydases and reductases secreted by the plant tissues (Tschirch, 1918). The evidence advanced by Haldane and Makgill (1923), however, is entirely in favour of Miehe's conception that bacteria are responsible for the earlier part of the heating, during which the temperature of the hayrick rises to 70 to 75° C.

Fermentation of Tobacco.

Though the question of the spontaneous heating of hay may appear of comparatively slight economic importance, the principle involved in this reaction is of great interest since it governs the fermentation of, and production of flavour in tobacco.

After drying (airing) the tobacco leaves are placed in suitable heaps and damped with steam or water to increase their moisture content to 20 to 25 per cent. The temperature of the heap increases as a result and may reach 70° C. This is excessive, however, and normally the temperature is maintained at about 50° C. by repacking the heap every third or fourth day. The repacking is usually repeated from five to ten times before the fermentation is complete. From the available information it would appear that the responsible bacteria resemble those isolated by Miehe from spontaneously heated hay, notably his *coli*-like type.

The changes which the fermentation is expected to perform comprise the decomposition of protein, fat and glucosides, and the elimination of sugars and hemicelluloses (Boekhout and de Vries, 1909, and Oosthuizen and Shedd, 1913).

It has not yet been convincingly proved that bacteria are responsible for the tobacco fermentation. Some writers, notably Vernhout (1900), and Koning (1900), maintain that they are, while others, Loew (1900), Oosthuizen and Shedd (1913), Jensen (1908), and Boekhout and de Vries (1909), are equally emphatic that bacteria cannot be responsible. Whether responsible or not for the changes which occur in the tobacco leaf during its fermentation, there can be no doubt that micro-organisms, including lower fungi, are present in large numbers during this stage of the preparation of the tobacco leaf. During the final manufacture of tobacco no special precautions are taken to prevent the surviving part of this microflora from developing. In fact, the addition of water to the raw tobacco during its preparation for smoking purposes, which becomes necessary owing to the brittleness of the leaf, creates conditions which may well give rise to renewed bacterial activity, and are known to do so. Mildewing of tobacco through the development of such fungi as *Aspergillus fumigatus* and *Aspergillus glaucus* is by no means an infrequent occurrence, and is at present difficult to control.

Ensilaging.

In the preparation of silage and of sauerkraut bacteria are now generally accepted as the causative agent. The processes taking place have been shown to involve the removal of soluble carbohydrates and part of the hemicelluloses of the treated vegetable tissues and the conversion of their proteins into amino-acids and peptones. As a result volatile and non-volatile products are formed, chiefly acetic and lactic acids and to a lesser extent ethyl alcohol and formic acid. Mannitol, according to Dox and Plaisance (1917), is another fermentation product met with in ensilaged fodder. Its origin can be traced to certain lactic acid bacteria.

The bacteriology of silage and sauerkraut has been the subject of several interesting investigations during recent years. Esten and Mason (1912) have shown that various types of yeast may develop in young silage during the first few days of its fermentation. Probably they take part in the breakdown of the available hexoses. According to Jones and Gibbard (1923) they soon give way to various types of bacteria, notably *coli*-like bacteria, and to lactic acid bacteria. The microflora of silage was found in Jones and Gibbard's investigations to increase from 30 millions per gramme of material before the fermentation to 260 millions after 14 days. The true lactic acid bacteria of the *B. bulgaricus* type rose from 9 millions to 200 millions per gramme. Gelatin-liquefying species, resembling or identical with *B. fluorescens liquefaciens*, decreased from 8 millions per gramme to half a million after 21 days of fermentation, undoubtedly owing to the unfavourable conditions created by the development of the lactic acid bacteria and to the increasing diminution of the pressure of the available oxygen.

Among the various bacteria found in silage, Fred, Peterson and Anderson (1921) regard the pentose-fermenting lactic acid bacteria as particularly important. With one of these strains, *B. lactipentoaceticus*, they were able to produce a normal silage from green maize stalks.

The work of many investigators, for instance, Samarani (1914), Sherman (1916), Swanson and Tague (1917), Dox and Plaisance (1917), and Edin and Sandberg (1921), confirm the importance of acid-producing bacteria and notably of lactic acid bacteria for the production of silage and sauerkraut. Lower and higher fungi apparently do not participate in these fermentation processes and are not usually found except on the exposed surfaces of the fermenting material.

Fermentation of the Cacao and the Coffee Bean.

Though both of these processes are of great and growing economic importance, the investigations which have been carried out have failed to establish the exact nature of the reaction taking place during the fermentation. It remains undecided even whether it involves the soluble carbohydrates of the pulp only, which surrounds the seeds of the cacao and the coffee plant, or whether it affects in addition the pectin and the hemicelluloses. The fermentation of the cacao bean has so far been most intensively investigated, and here again some workers, Preyer (1913) and Nicolls (1913), attribute the changes taking place to a yeast, *Saccharomyces theobromæ*, which Preyer isolated from fermenting cacao beans. Nicolls admits the participation also of acetic-acid-producing bacteria.

As the liquid draining from the fermenting beans has been shown to contain both lactic and butyric acid, in addition to acetic acid and ethyl alcohol, it is almost certain that other micro-organisms than those mentioned above must participate in the fermentation. It is questionable even, as pointed out by Knapp (1920), whether the yeast present can take a major part in the reactions once the temperature of the fermenting beans has risen beyond the optimum of these organisms. This rise occurs at an early stage of the fermentation.

Other investigators of the cacao and the coffee fermentation, notably Loew (1913), Schulte im Hofe (1913), and Brill (1915), make plant enzymes responsible, though they do not deny the actual presence in the fermenting bean pulp of yeast and bacteria. The process of fermentation resembles that of tobacco: the fresh beans, still embedded in a sweet pulp, are collected in heaps and allowed to ferment, whereby the temperature of the heap rises to 45° C. or even 50° C. The high temperatures destroy the embryo of the bean, the tannic substances are converted into insoluble compounds, and the pulp is rendered more easily removable by washing.

THE DECOMPOSITION OF GLUCOSIDES.

The Indigo Fermentation.

That the hydrolysis of the glucoside indican and its conversion into natural indigo is due to the activity of bacteria was suggested by Alvarez (1887), but was ascribed subsequently to the function of plant enzymes secreted by the leaves of the indigo plants (Bréaudat, 1898); though it was admitted (Bergtheil, 1904) that bacteria capable of hydrolysing indican were invariably present in the infusion of fresh leaves which is used industrially for the isolation of indigo. After many years of ascendency the theory of plant enzymes as the active agency in indigo fermentation is losing ground, and it is becoming increasingly evident (Davis, 1921) that the presence of certain *coli*-like bacteria is essential for the successful production of natural indigo.

THE FERMENTATION OF STARCH AND SUGAR.

The utilization of starch and sugar for the preparation by fermentation of compounds of economic importance has been considerably extended during recent decades. It is unfortunately impossible in the available space to do more than briefly to refer to some of the more important developments which have taken place. At the end of this section a reference will be made to certain bacteriological problems of the sugar industry.

Power Alcohol Production.

An interesting aspect of the production of power alcohol is the utilization for this purpose of the hemicelluloses which occur in most vegetable waste. In the process patented by Norbury and his associates

(1924) waste, such as straw, grasses, maizecobs or husks are used, and the insoluble hemicelluloses are converted into soluble pentoses by treatment with acid. The resulting mash is fermented by a pentose-fermenting bacterium such as *B. acetoethylicus* Northrop (1919), under conditions which ensure a favourable development of the organism, notably a suitable hydrogen-ion concentration. The volatile fermentation products consist of a mixture of acetone and ethyl alcohol in yields equivalent to 33 to 35 per cent. of the hemicelluloses fermented.

With sufficient raw materials available the method would appear to offer prospects of a cheapened supply of motor fuels in countries where petrol has to be imported from afar, since the yield quoted above should insure an output of liquid fuel from waste grasses equal to or perhaps even superior to that obtainable from potatoes (Thaysen and Galloway, 1928).

The Butyl Alcohol Fermentation.

This is a technical application of bacterial activity which owes its success to the world war, but which, since then, has become increasingly important owing to the valuable solvent properties of its chief fermentation product. It was initiated by Fernbach and Strange (1912) and Weizmann (1915) with a view to increasing the available supplies of acetone, another important product of the fermentation. The bacteria responsible for the production of butyl alcohol and acetone from starch and hexoses are closely related to *Clostridium butylicum* Donker (1926), and occur fairly commonly in nature in soil and in decaying plant material. In addition to the formation of the two products mentioned, the breakdown of hexoses by this bacterium results in the formation of appreciable quantities of hydrogen and carbon dioxide, as well as of some ethyl alcohol. The approximate proportions of its chief fermentation products are *n*-butyl alcohol 25·0 per cent.; acetone 10·77 per cent.; 62·61 per cent. of carbon dioxide and 1·6 per cent. of hydrogen (Reilly and collaborators, 1920). The bacteriological aspect of the butyl alcohol fermentation was dealt with in some detail by Thaysen (1921). It is emphasized in Thaysen's paper that one of the most serious sources of infection met with in this industry is one caused by lactic acid bacteria, an infection which in the course of a few hours is able completely to arrest the development of the butyl alcohol bacteria. The presence of a bacteriophage in faulty fermentations has been suggested by Legg (1926).

A somewhat analogous fermentation process, though complicated by the presence of yeast and various bacteria, forms the basis of the drink 'Chicha' which is extensively consumed in certain South American countries.

The Butyric Acid Fermentation.

Though butyric acid has been produced by fermentation for many years, there is practically no information available on the methods used. There is little doubt that these methods are still of the most primitive nature, similar to those employed in the early days of lactic acid manufacture, when the fermenting mashes frequently yielded butyric acid instead of lactic acid.

Many species of butyric acid bacteria have been described in the course of time. The typical form is an anaerobic spore-forming rod, which ferments hexoses with the production of butyric and acetic acid in proportions similar to those yielded by the butyl alcohol bacillus. Varying small percentages of butyl alcohol and acetone are usually produced. This organism possesses the property of fermenting lactates with the formation of butyric and acetic acids.

The Lactic Acid Fermentation.

The first method for the production of lactic acid was described by Boutron and Fremy (1841) at a time when casein was thought to be the agency responsible for the conversion of hexoses into this acid. With the realization of the functions of bacteria great improvements were introduced into Boutron and Fremy's method, notably by Avery (1882) and Claflin (1897), though the use of authentic pure cultures of lactic acid bacteria was neglected even in their methods. To-day, modern lactic acid factories usually employ types of lactic acid bacteria related to or identical with *B. acidificans longissimus* Lafar (1896), a typical representative of the true rod-shaped lactic acid bacteria, frequently described under other names, such as *B. Delbrücki, B. bulgaricus,* &c. They readily ferment maltose, saccharose (molasses), and glucose (from maize and other cereals), at the temperatures of 45 to 50° C., temperatures at which the development of butyric acid bacteria is delayed or prevented. By the use of such types of lactic acid bacteria the danger of infection of the mashes is considerably diminished and better yields of lactic acid are consequently obtained. In modern factories yields should be possible of as much as 98 per cent. of lactic acid calculated on the carbohydrates fermented.

In the modern lactic acid fermentation plant, as in those of all other fermentation processes, the previously sterilized mash, containing in this case 10 to 15 per cent. of carbohydrates, is inoculated under aseptic conditions with a pure culture of the active bacteria and then allowed to ferment at the optimum temperature. The acid formed is neutralized at intervals with sterile suspensions of calcium carbonate or lime. The fermentation is completed in from four to six days, and the calcium lactate is then recovered from the mash by evaporation and crystallization.

Lactic acid finds an increasing market, notably in the textile industry, as a reducing agent, and in the leather industry for the softening of hides.

The Bacteriological Problems of the Sugar Industry.

At three stages in the manufacture of cane and beet sugar, the invasion and the development of bacteria is likely seriously to interfere with the workings of the sugar factory. Of these, two are more or less restricted to the factories working with sugar cane, while the third is of particular importance to beet sugar factories.

In the cane sugar factories the freshly pressed juice forms a particularly favourable medium for many bacteria and fungi before its suspended particles have been removed by coagulation. Signs of bacterial activity may be noticeable after two hours standing of the juice, resulting in the inversion of a smaller or larger percentage of the dissolved saccharose, a change which seriously affects the subsequent recovery of the cane sugar by crystallization.

Inversion of saccharose is the chief danger also at the second stage, during which bacteria and fungi interfere with the manufacture of cane sugar. Where the raw sugar is stored for any length of time it is liable to 'sweat'. According to van der Bijl (1921) the surrounding temperature, the moisture content of the sugar, and the humidity of the atmosphere are contributory causes to this sweating, which creates conditions favourable to the development of bacteria and fungi. Van der Bijl isolated three types of bacteria from sweating sugar; they were related to *B. mesentericus*. Several types of *Aspergillus* and *Penicillium* were also obtained. Both the bacteria and fungi were capable of inverting saccharose.

More serious from a hygienic point of view is the third bacteriological problem of sugar manufacture, the pollution of the effluents of sugar factories through the development of *Leptomitus*, *Sphaerotilus* and related sheath-producing bacteria. Originally it was maintained, (Cohn, 1881 and 1884), that the direct cause of the excessive development of these bacteria, a development which frequently results in the complete blocking of the neighbouring streams, was due to the sugar contained in the effluent of beet-sugar factories. Subsequent investigations by Kolkwitz (1903) failed to confirm this, and attributed the pollution to the organic nitrogen present in the effluent. Kolkwitz's conclusions have recently been queried, however, and the latest investigations have substantiated Cohn's conclusions as to the part played by saccharose in the development of *Leptomitus* and *Sphaerotilus*. There remains to be solved, however, the very important problem of the prevention of this fouling of sugar-beet factory effluents.

THE DECOMPOSITION OF ORGANIC NITROGEN COMPOUNDS.

In drawing attention to this field of economic bacteriology, the writer would like to refer to two instances by way of example. Ehrlich (1914) has shown that amino-acids are decomposed by yeast or by fungi in the presence of fermentable carbohydrates. In this way phenylalanine, for instance, is converted into phenylethyl alcohol, an important part of the essential oil of roses possessing the flavour of rose oil. It is highly probable that by the adoption of a bacteriological method this substance could be more economically prepared than by the distillation of rose petals. In the degumming of natural silk attempts have been made from time to time to utilize tryptic enzymes instead of the now generally employed soap treatments. In the writer's experience bacteria exist which are capable of dissolving sericin, and therefore of degumming raw silk without the use of chemicals. It remains to be shown whether a technical process for the degumming of silk can be based on the activity of bacteria.

FURTHER ASPECTS OF THE APPLICATION OF BACTERIA.

As knowledge increases, other applications of bacteria to industry will no doubt be found. A fairly recent application is that in which Helbronner and Rudolfs (1922) have used certain sulphur bacteria for converting naturally occurring zinc minerals into zinc sulphate under conditions which might make it possible to utilize this agency for the recovery of zinc from its natural deposits.

The Bacteriological Aspects of the Formation of Minerals.

Two problems falling under the above heading will be discussed here: the deposition of iron ore and the formation of coal and its allied substances, lignite, peat and natural oil.

THE DEPOSITION OF IRON ORE.

Ehrenberg (1836) first drew attention to the possibility that micro-organisms might participate in the natural deposition of iron ore, and reported that he had observed an ochre-coloured mud in water-filled holes of bogs, which on microscopic examination could be shown to consist of threads of an organism containing iron oxide in its cells. Subsequently Ehrenberg's organism, or at least the one referred to above, was identified with Kützing's *Chlamydothrix ochracea*, a typical iron bacterium. This, and other iron bacteria subsequently described, belong to a group of micro-organisms intermediate between the *Bacteriaceæ* and the *Algæ*, which secrete a sheath holding the individual cells in position as a chain of rods, frequently fixed to solids in the surrounding medium. The iron bacteria possess the remarkable property of being able to oxidize ferrous salts to ferric oxide, which they deposit in their sheath. They occur extensively where ferrous salts are available in solution, and are responsible (Adler, 1904) for the destruction of iron-containing mineral waters. More serious is their action on water conduit pipes, which they frequently cover and finally block with a layer of rust. Their activity in this direction has been studied by several investigators, notably by Schorler (1906), and in this country by Ellis (1919). Examined under the microscope this rust can be shown to consist of the sheath of iron bacteria filled with iron oxide. Where the rust had been deposited years before the examination, the sheaths may not be readily discernible.

Similar conditions occur in the accumulation of bog iron and other natural iron deposits, and Winogradski (1888) did not hesitate to express the opinion that these deposits were formed by the accumulation of iron bacteria. Molisch (1910), who failed to trace the remains of iron bacteria in more than a fraction of the samples of iron ore examined by him, did not accept Winogradski's statement unconditionally, but was prepared to admit that in some cases iron bacteria might have participated in the deposition of iron ore. Even recent ochre deposits were declared by Molisch to have been frequently formed in nature without the participation of iron bacteria. This, however, has not been confirmed on subsequent investigation. Harder (1919), for instance, detected the remains of iron bacteria in all the ochre deposits examined by him. Cholodny (1926) confirmed Harder's views. The general consensus of opinion now undoubtedly accepts iron bacteria as the responsible agent in the accumulation, not only of ochre, but of all iron ore deposits. The absence of traces of bacteria in many of these ores is considered to be due to their disintegration in the course of time.

THE FORMATION OF COAL.

It is generally accepted to-day that coal and substances such as peat, lignite, oil, and natural gases owe their origin to the decay of the vegetation of past and present ages, and that the peat bog of to-day represents the conditions under which this decay proceeded. It is to be concluded therefore that coal, and probably oil, were in most cases deposited as peat.

An interesting description of the conditions of the early stages of coal formation was given by White and Thiessen (1913) in their account of the peat swamp of eastern Sumatra, which covers more than 80,000 hectares. In this swamp there is being deposited a high-grade peat, reaching a depth of nine metres and containing comparatively little ash. The swamp is covered by about 60 cms. of tea-coloured fresh water in which grows a dense forest of mixed evergreen trees, forming a canopy over the swamp, and whose leaves, dead twigs and trunks fall into the water. The trees stand so close together that their roots form a matted tangle at the surface of the peat.

The rate of formation of peat in this swamp is thought by White and Thiessen (1913) to be considerably faster than in bogs of colder climates. Normally some ten years are required to produce 30 cms. of young peat, but continued decomposition will be responsible for a decrease and, at a depth of 4·5 to 6 metres, this amount of accumulated debris may have been reduced to a layer of only 2·8 cms. At that rate 30 cms. of peat, at a depth of 4·5 metres, would take about 100 years to form.

From the above outline of the conditions of decay in a typical peat bog, it will be seen that peat, and consequently coal, and perhaps oil, was and is formed under conditions which favour the development of bacteria, at least as far as temperature and moisture are concerned. As the vegetable debris rapidly becomes water-logged in the peat bog, there is little likelihood of fungi taking an extensive part in its destruction, since fungi, as already mentioned, do not develop well under moisture conditions exceeding 60 per cent.

It is not surprising, then, that bacterial activity should have been connected with the formation of peat and coal. The first observations in this direction were made by van Tieghem (1879), who maintained that in samples of coal he had detected signs of the presence of *Amylobacter* —at the time regarded as a cellulose-decomposing bacterium. His observations were subsequently extended by Rénault (1895–8), who described a number of bacteria observed in coal. More recently Thiessen (1913), and Grüss, according to Lessing (1923), have observed fungus spores and fungus mycelium in coal, and White and Thiessen (1913) have reported the presence of living bacteria in a peat bog more than 800 cms. below the surface. The latter observation, however, cannot be accepted as correct without additional evidence, seeing that bacteria, at a depth of 800 cms. below the surface, would have had to remain alive for some 2,000 years, the time required to form this layer of peat.

Basing their deductions chiefly on Fremy's view of the occurrence of a 'fermentation tourbeuse', Fischer and Schrader (1922) have developed a theory on the formation of coal in which it is maintained that the plant tissues decaying in a peat bog lose the whole of their cellulose content through the action of fungi, while the lignin remains unattacked and represents the mother substance of peat and coal. Of special interest to the bacteriologist is the question whether bacteriological data can be supplied in support of Fischer and Schrader's theory, which at present is widely accepted. Unfortunately this is not the case. Apart from the observations already mentioned there is no bacteriological evidence in support of, but very serious evidence against, it. Reference has already been made to the unlikelihood of fungi taking an active part in the decay of plant debris under water-logged conditions. And as regards the participation of bacteria, notably cellulose-decomposing bacteria, Thaysen and his collaborators (1926) have shown that bacterial activity comes to a standstill in a peat bog long before the cellulose of the decaying plants has been destroyed; in fact, while a very large percentage of the debris is still intact. They conclude from their investigations that other agencies than bacteria must be responsible for the decomposition of the bulk of the decaying vegetable matter, but recognize that bacteria may have participated in the earlier stages of peat formation, and possibly may have accelerated the subsequent non-bacterial decay. Further, they show that though decomposed, the cellulose is not completely destroyed, as suggested by Fischer and Schrader, but can be detected as part of the humus in the final peat.

With this restriction on the activity of bacteria in peat and coal formation, the question arises whether the natural gas, largely methane, which escapes from bogs and coal seams, can be a product of bacterial activity. In the case of coal and peat deposits this question must at present remain open. In the case of the evolution from the silt of ponds and lakes, there can be no doubt that Omelianski's (1895) cellulose-decomposing bacteria *B. methanigenes* and *B. fossicularum*—as well as other related types—are the chief agents in the production of the gas, which occasionally accumulates in such quantities that it can be tapped for technical purposes.

As regards the participation of bacteria in the formation of oil-bearing strata no direct evidence has up till now been supplied.

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CHAPTER II. THE BACTERIOLOGY OF WATER.

BY H. HENRY (BIRMINGHAM).

The Bacteria which Occur in Water.

THE bacteria which are to be found in water may be classified into two main groups. Of these, the first comprises all the organisms which normally exist in water or in the surface layers of soil, while the second embraces those which indicate fæcal pollution.

The first group may be briefly set out as including the following subgroups:

1. *Fluorescent bacteria.* The organisms of this group are characterized by the production on artificial media of growths which possess a green fluorescent shimmer. They are perhaps the most widely distributed of all the organisms occurring as normal inhabitants of water, and *B. fluorescens* may be looked on as a typical representative.
2. *Chromogenic bacteria.* Of those producing red pigment, the most frequent are *B. prodigiosus*, *B. ruber* and *B. indicans*. Orange-coloured or yellow colonies are due to *B. aquatilis*, *B. aurantiacus* and *B. fulvus*, &c., while at certain seasons, more especially in river water, violet-producing organisms such as *B. violaceus* and *B. cæruleus* may be common.
3. *Proteus group.* These produce a thin, slimy, continuous surface film on solid media, and often give rise to an ammoniacal odour. The group includes types which occur frequently in putrescent organic matter.
4. *Sporing bacteria.* The best known of these are *B. subtilis*, *B. mycoides* and *B. mesentericus*. They are normal inhabitants of the surface layers of soil, and are found in greatest abundance in spate water, when heavy rain has fallen after a period of dry weather. They produce large, opaque, vigorously-growing colonies.
5. Natural waters often show bacteria, which are not referable to any of the groups just mentioned. They can only be described as being non-fluorescent, non-chromogenic, non-sporing, and do not give rise to growths resembling *Proteus*. Nor do they give the fermentation reactions of members of the *coli* group.
6. *Cocci.* Most of the cocci occurring in water are not chromogenic. *M. candicans* and *M. aquatilis* are the most common species.

The organisms which have just been enumerated as being normal inhabitants of water are of little significance as compared with those of intestinal origin, for the latter indicate pollution with sewage, and by their presence bespeak the possibility of contamination by those disease-producing organisms which are known to occur in human fæces.

Although the suspicion of water-borne disease must have occurred fairly early in the history of civilized human communities, it is only in comparatively recent times that our knowledge in this respect has enabled us to escape from the disadvantages suffered by our predecessors. Our practical interest in the subject, as seen from its bacteriological aspects, may be said to date from the occurrence of the Hamburg cholera outbreak of 1892, and there can be little doubt but that this event did much to supply the initial incentive for all subsequent bacteriological investigations on the problem of a clean water supply.

As evidence of pollution by excreta, bacteriologists are accustomed to examine samples of water for bacteria of the *coli-aerogenes* group. In addition, it may be necessary to look for fæcal streptococci and for *B. welchii,* but it may be said that at the present day the search for these latter is usually omitted.

Some Factors which influence the Bacterial Content of Water.

Water, as it exists in the clouds, is probably quite sterile, but as it falls to the earth in the form of rain it becomes contaminated with organisms which are suspended in the atmosphere. Rain-water in a city thus shows a higher bacterial content than it does in the open country. For the 1883–6 period, Miquel (1886) recorded 4 organisms per c.cm. in rain-water collected at the Montsouris observatory as against an average of 19 per c.cm. for samples collected inside Paris. After contact with the soil rain becomes heavily polluted with the numerous bacteria which it washes away from this source. When it collects in pools and rivers it is described as *surface water.* If it becomes a *stored water* in a loch or reservoir there may result a considerable purification by sedimentation, and this may be still further enhanced if it be filtered by natural gravitation through soil and rock, in which case it is known as *ground water.*

In its bacterial content surface water shows great variations, most of which are incidental to rainfall. The first effect of rain, especially if it comes after a long period of drought, is to increase pollution by the addition of soil washings, and, in fact, most rivers containing water of good quality are found to yield higher bacterial counts during the wet seasons of the year than in dry weather. On the other hand, the addition of surface washings to a heavily polluted river may result in apparent purification by actual dilution. A table which is to be found in Prescott and Winslow (1924), and which is compiled from data obtained by Kisskalt in respect of the Lahn, a river of good quality, and the Wieseck, a polluted stream, illustrates well this differentiating effect.

For descriptive purposes it is necessary that the factors which determine the increase or decrease of bacteria in water be considered separately, but it is naturally quite impossible to assign to any one factor its due measure of influence, because of the simultaneous interaction of all factors together.

Perhaps the most important factor influencing the survival and multiplication of bacteria in water is its content in food supply. It is to be noted, however, that in their metabolism these organisms are very diverse in character. Probably the least exacting are the nitrifying organisms of Winogradsky (1890) which will live in the absence of all organic matter. There must be many other types also which closely approach these last in their minimal nutritional requirements, for it would be difficult to discover under natural conditions samples of water which are completely free of bacteria. More than half a century ago, Burdon Sanderson (1871) found evidence of bacteria in distilled water which had been left open to the air. Even the merest trace of organic material would seem to be sufficient to induce growth of certain of the more commonly occurring forms of bacterial life. On the other hand, it is a frequent observation that waters which contain a large amount of organic material, whether it be of animal or of vegetable origin, not infrequently support a large bacterial population. Where the organic content remains at a high level, as in heavy pollution with sewage, the organisms that are particularly adapted to a parasitic existence in the bodies of animals and plants may survive for some time, but with dilution and oxidation they tend to die out and disappear, leaving only the ordinary harmless water bacteria to take their place.

The question of the influence of *temperature* on the bacterial flora of water has been very extensively studied. If a sample of water be kept at room temperature the bacteria are usually found to multiply at a very rapid rate. For instance, Frankland (1894) showed that a count in distilled water might increase sixfold after 6 hours at room temperature. Whipple (1901) finds that in most cases the first effect of storage is a slight reduction, followed after 24 to 48 hours by a very great increase. Miquel (1891), as the result of a long experience, concluded that it was actually possible to differentiate between spring waters and impure waters, in that the former were characterized by a rapid but transitory power of multiplication, whereas with polluted waters the multiplication is slow and persistent. It must be assumed that increase is likely to occur only where food supply is sufficient, so that in the case of potable waters the increase affects only the real water flora. On the other hand, the conditions necessary for survival and growth of the intestinal organisms do not obtain in a good quality water, and in this case the effect of increase in temperature is exactly the reverse of that just described. Hale and Melia (1910) found that in an artificially inoculated water, *B. coli* lived for 8 to 10 days at 37° C., for 38 to 75 days at 20° C. and 77 to 84 days at 8° C. A still more marked lethal effect is manifest in the case of typhoid bacilli, as was shown by Houston (1911), who observed that after a week at 0° C. 46 per cent. remained, at 5° C. 14 per cent., at 10° C. 0·07 per cent., while at 18° C. only 0·04 per cent. were still viable. A similar result showing the inhibitory effect on typhoid bacilli of summer as compared with winter temperature is also reported by Ruediger (1911). Cohen (1922) correlated the temperature coefficients of *B. coli* and of *B. typhosus* and finds the comparative resistance at 0° C. to be as 67 to 1, while at 30° C. it is as 8 to 1. In connection with the apparent preservative effect of cold on typhoid bacilli, Prescott and Winslow (1924) make the following comment: 'Almost without exception outbreaks of typhoid fever due to polluted water occur in cold weather and this is in part at least due to the greater persistence of typhoid bacilli at low temperatures'.

The subsidence in still water of suspended matter including bacteria, which constitutes the process of *sedimentation*, is universally conceded to be one of the most potent factors in the natural purification of water. Striking figures which show the beneficial effect of storage are recorded by Frankland (1894), by Houston (1909) and by many others. The process naturally reaches its maximal effect in the case of lochs or reservoirs, but it is often found to be equally well pronounced in running streams. The method is used not only for domestic supplies but also in the disposal of sewage and refuse liquids. Further, as it has been observed that the rate and degree of purification depend on the amount of suspended matter initially present and the rapidity with which its subsidence is brought about, the process of sedimentation can be artificially accelerated by the addition of precipitants.

Certain waters have been found to possess a *bactericidal action* in themselves, a phenomenon first investigated by Miquel (1891) and later by Hankin (1896). Miquel discovered that a water which has supported the multiplication of a particular organism became destructive to it when it was reintroduced, and ascribed this result to the formation of soluble and toxic products which inhibited growth. These products he found to disappear on boiling and to be capable of concentration by evaporation at 30 to 35° C. Hankin's experiments in regard to the water of the Jumna showed that bacterial content of the river decreased from 100,000 to less than 100 per c.cm. over a distance of 5 kilometres. He observed also that the river was markedly bactericidal for cholera vibrios, and his results have been cited by d'Herelle (1922) as the first recorded instance of bacteriophage action. It may be said, however, that it is doubtful whether Hankin's findings are explicable on this basis.

On the effect of *insolation* as a water-purifying agent, opinions are at present divided. Our first information in regard to the bactericidal effect of sunlight is to be found in the classical researches of Downes and Blunt (1877, 1878). These investigators demonstrated also that the blue-violet end of the spectrum was responsible for the lethal action of sunlight, and explained the effect as being due to a gradual oxidation which damages the bacterial protoplasm. The effect of light on bacteria furnished a subject which attracted most of the best bacteriologists of the period, and an excellent survey of the earlier literature dealing with the problem is given by the Franklands (1894). In the case of Indian rivers and lakes, Clemesha (1912) has claimed sunlight to be a powerful purifying agent, but it is to be noted that in emphasizing this factor he tends rather to ignore the effect of sedimentation.

The Bacteriological Examination of Water Supplies.

The first concerted action on the standardization of methods which deal with the bacteriological examination of water supplies was taken by the American Committee of Bacteriologists (1898). This body became a permanent committee in 1901, and since that date it has published a long series of important memoranda concerning the bacteriological aspects of water and of sewage. Problems of a very similar character have been dealt with in this country by Committees which were appointed by the Royal Institute of Public Health and which published their findings in 1904 and again in 1914.

The bacteriological examination of water comprises two operations: firstly, the enumeration of the number of organisms present, and secondly, the detection and measurement of the number of excretal organisms it contains.

THE QUANTITATIVE ESTIMATION OF BACTERIAL CONTENT.

Water in accurately measured amounts is added to gelatin and agar, each medium having a pH of 7·0 as ascertained by phenol red or bromthymol blue. The gelatin plates are incubated at 20° C. in a moist incubator for 2 days, while the agar plates are kept at 37° C. for 24 hours. In the former case the count provides a comparative index of the soil and water organisms present, whereas the agar count gives a rough estimate of the intestinal bacteria existing in the sample. The English Committee suggested that the ratio of the 20° C. count to the 37° C. count provided a figure of some importance; in that with good waters the figure is usually much above 10, whereas in the case of pollution the figure approaches 10 and may often be considerably less. Although this statement is in the main correct, there are certain circumstances in which it is to be accepted with considerable reserve. For instance, Race (1916) has shown that the ratio is affected by the general outdoor temperature for the time of year, being low in summer and high in winter. With this observation are to be correlated the earlier findings of Horrocks (1901) in respect of tropical surface waters which may contain large numbers of *B. fluorescens* growing better at 37° C. than at 20° C. Again, in the case of chlorinated waters the ratio frequently approximates to 1, as shown by Clarke and Gage (1909) and by Tulley (1912), the probable explanation in this case being that it is the spore formers which survive chlorination, and which develop just as readily at 37° C. as at 20° C. Quite apart from the question of ratio, however, the 37° C. and the 20° C. counts, when considered separately, may provide valuable information, especially if they are carried out at regular intervals over long periods of time, in which case marked irregularities in the bacterial content of a particular water are the more easily detected.

THE DETECTION AND ENUMERATION OF THE EXCRETAL ORGANISMS.

The Colon Group of Bacteria.

Water in a geometric series of quantities is added to bile salt lactose peptone water. The development of acid and gas in two days is suggestive of the presence of a colon bacillus. The test is almost universally known as the 'presumptive test', and was originally carried out with glucose-containing media, as this sugar was said to be more sensitive than lactose. It was soon discovered, however, that many organisms occurring in water and yielding a positive test with glucose are not of the colon group. Even with lactose numerous instances have been reported of spurious presumptive tests, these being due to *B. welchii*, as recorded by Frost (1916), and by Levine (1921); to *B. subtilis*, as reported by Meyer (1918), and Ewing (1919); or to the combined action of two organisms as suggested by Sears and Putnam (1923).

Although most bacteriologists are now agreed that lactose is a necessary ingredient of the 'presumptive test' medium there is no such unanimity in respect of the use of bile salt. In fact, it is to be noticed that the American Standard Methods Report for 1923 advise lactose broth without bile for the presumptive test. On the other hand, Stokes and Stoner (1909), using as media dextrose broth, lactose broth, and lactose bile, found the last medium to give the most reliable results, 95 per cent. of all colonies obtained from it being of the coli-aerogenes group. Jackson (1906, 1907), too, is convinced that bile gives the best results, an opinion based on the examination of 5,000 samples at the Mount Prospect Laboratory. Prescott and Winslow (1924) affirm that lactose bile is of the greatest use in dealing with heavily polluted water, but that the addition of bile does diminish positive results in the case of waters of fair quality. There can be little doubt but that discordant opinions in this matter are due to the difficulty of measuring such a variable product as bile salt, and hence of determining the concentration which will produce the best selective effect.

In addition to the use of bile salt several other enrichment methods have been employed with a view to encouraging the growth of colon bacteria at the expense of competing organisms. Of these there may be mentioned: phenol (Irons, 1902; Reynolds, 1902), neutral red (Rothberger, 1898; Savage, 1901), æsculin (Harrison and van der Leck, 1909) and incubation at 46° C. (Eijkman, 1904). Also, as a sequel to the investigations of Churchman, the use of triphenylmethane dyes as differential antiseptics has received much attention in recent years at the hands of water bacteriologists. The elimination of spurious results of the presumptive test caused by anaerobic gasformers is said to be effected by the addition to a lactose medium of gentian violet (Hall and Ellefson, 1918). A similar effect is claimed for brilliant green by Muer and Harris (1920), and Bronfenbrenner, Schlesinger and Soletsky (1920) claim that rosolic acid inhibits all Gram-positive organisms, while allowing the Gram-negative bacteria to grow freely. In discussing the results obtained by these workers, Prescott and Winslow (1924) suggest that investigations with selective dyes show great promise of yielding a more reliable and satisfactory presumptive test for organisms of the colon group.

In the presence of a positive presumptive test it is necessary to obtain confirmatory evidence of the existence of a true colon bacillus. A small portion of the culture from the lowest dilution which has yielded acid and gas is therefore plated out on MacConkey's medium, and red colonies isolated from these plates are inoculated into lactose peptone water and also on to agar tubes. The formation of acid and gas in the lactose tube, and the appearance in smears taken from the agar surface of a Gram-negative non-sporing bacillus afford definite and confirmatory evidence. For the actual isolation of a colon bacillus by means of plates several media other than MacConkey's litmus lactose agar are available. Endo agar is used very extensively in America, and Levine (1921), who employs an eosin-methylene blue agar claims that colonies of *B. coli* and of *B. aerogenes* can be easily differentiated on this medium. As far as the water bacteriologist is concerned the colon group is divided into two different types. In this country these have been designated rather broadly as 'typical' and 'atypical' *B. coli*, whereas in American literature they are referred to as the *B. coli* type and the *B. aerogenes* type. The first clear evidence of the differentiation between these two types is to be found in the pioneer work of MacConkey (1900, 1901, 1905, 1906), who demonstrated that the *B. aerogenes* type, which was saccharose-positive and dulcitol-negative, did not produce indole and reacted positively to the Vosges-Proskauer test. These findings have been repeatedly confirmed. The next step in the differentiation of these types we owe to Rogers and his colleagues in the Department of Agriculture in Washington, who showed that the fermentation of dextrose resulted in the production of carbon dioxide and hydrogen in equal proportions in the case of organisms of the coli type, whereas the ratio of carbon dioxide to hydrogen was usually 2 to 1 or more in the case of the aerogenes group (Rogers, Clark and Davis, 1914). A further differentiation was effected by the researches of Clark and Lubs (1915) who found that the types mentioned produced different hydrogen-ion concentrations when grown in suitable media, and were able to demonstrate this by means of methyl red, the coli type giving an end reaction of pH 4·8 to 5·6, while the aerogenes type reached a pH of 5·8 to 6·6 under similar conditions. A further valuable test would seem to be that described by Koser (1918) who has found that *B. aerogenes* is capable of utilizing uric acid as a source of nitrogen, whereas *B. coli* is incapable of doing so. The tests, therefore, which would appear to be of practical value in distinguishing *B. coli* from *B. aerogenes* are the indole test, the Vosges-Proskauer reaction, the methyl red test and the Koser test, the reactions being tabulated as follows:

	Indole	*Vosges-Proskauer*	*Methyl red*	*Koser*
B. coli	+	—	+	—
B. aerogenes	—	+	—	+

It must be said, however, that these correlations are not absolutely constant and that aberrant types are not infrequent. Organisms of the coli type may be still further defined by their sugar reactions as including *B. coli communis*, *B. coli communior*, *B. acidi lactici* and *B. neapolitanus*, whereas organisms of the aerogenes type are similarly represented by *B. lactis aerogenes* and *B. cloacæ*. That the differentiation just cited is of practical significance may be deduced from the large mass of evidence that is now available in respect of the habitat and source of the two types which have been discussed. The coli type is that most commonly found in human and animal fæces, while the aerogenes type occurs chiefly as a saprophyte in soil and on cereals. In this connection the reader is referred to results published by Chen and Rettger (1920) Wood (1920) and Levine (1921). The last-mentioned observer discusses the problem at length and gives a valuable summary of results gathered from a number of sources. This is here given in tabular form:

Colon bacteria isolated from	*Number of strains*	*B. coli. type per cent.*	*B. aerogenes type per cent.*
Human fæces	2,534	94·1	5·9
Animal fæces	1,832	92·6	7·4
Soil and cereals	1,141	13·5	86·5

It becomes apparent, therefore, that the finding of an organism of the coli type in a sample of water affords clear indication of recent sewage pollution, whereas the discovery of an organism of the aerogenes type, which is apparently derived from soil and cereals, is to be interpreted as less serious. It is to be noted, however, that the finding of a few *B. coli* in large samples of water, or even their occasional presence in small samples, has not necessarily any significance. On the other hand the detection of *B. coli* in a large percentage of small samples is very definitely an indication of recent fæcal pollution. Thus the detection of *B. coli* in a water assumes importance only where the result indicates the number of *B. coli* present in a given volume of the sample. That such an enumeration is absolutely necessary was first pointed out by Theobald Smith (1893). To reach a quantitative estimation the method most commonly followed is that based on the 'coli index', which is calculated as the reciprocal of the smallest amount of water that yields a positive confirmed presumptive test. Thus, if 0·1 c.cm. of a water sample gives a positive test the 'coli index' is expressed as being 10 per c.cm. or as 1,000 per 100 c.cm. That the results as thus expressed fail to yield a reliable figure is indicated by the work of McCrady (1915), Greenwood and Yule (1917), Reed (1925) and others, who have suggested a series of formulæ based on the theory of probability. An interesting application of Reed's formulæ as applied to consistent and anomalous findings is quoted by Streeter (1927).

The Fæcal Streptococci.

The streptococci present in water samples can best be isolated by growth in dextrose or lactose broth for 36 to 48 hours, followed by plating on litmus lactose agar, on which most types give small, intensely red colonies. They are not easy to define, but may be said broadly to exhibit the following common characters. Some show development in well-marked chains, but there are many types which are to be found in pairs or irregular groups. On agar slopes most strains produce slowly growing, rounded, transparent colonies, but in stabs under less aerobic conditions, growth is as a rule much more vigorous. Most are lactose fermenters, do not form indole, and, with few exceptions, do not liquefy gelatin.

The interest of the streptococci to the water bacteriologist lies in that their study at one time appeared to offer a means of distinguishing human from animal contamination. It may be said, however, that the fermentative characters of the streptococci of human, equine and bovine origin, on which this hope was based, are by no means constant, and do not, therefore, provide a reliable criterion for successful differentiation.

In the case of potable waters which give a positive coli test the search for streptococci is very frequently negative, even when considerable quantities are used for test purposes. Savage and Wood (1918) found that under experimental conditions the disappearance of colon bacteria and of streptococci ran parallel, though it was more rapid in the case of the latter. The same general parallelism is to be noted in a percentage of dotable surface waters, the streptococci being, however, at a much lower numerical level than colon bacteria (Savage and Read, 1917). In most laboratories the search for streptococci has been abandoned as being less delicate than the coli test, and it is only under exceptional circumstances that a positive streptococcus test in the absence of the colon bacillus can be accepted as providing evidence of fæcal pollution.

The Fæcal Anaerobes.

The common anaerobes of the human intestine, viz. *B. welchii* and *B. sporogenes*, are almost ubiquitous, and as they are capable of resisting the physical conditions to which the other fæcal organisms succumb, they may be discovered in polluted water long after all other traces of contamination have disappeared. In the case of potable waters these organisms are usually present in very small numbers, so that, to demonstrate their presence it may be necessary to use large quantities for test purposes (Horrocks, 1901). In actual practice it is usual to pass from 2 to 10 litres of water through an earthenware filter under low pressure, to make a suspension in small bulk of the organisms thus separated out, and to plant graduated fractions of this suspension into tubes of sterilized fresh milk, which are then heated to 80° C. for 20 minutes. The development of the stormy fermentation which is characteristic of *B. welchii* provides sufficient evidence of the presence of this organism. On the other hand, if small quantities of ferric chloride be added to the milk, the presence of *B. sporogenes* is shown by the slow and gradual blackening of the whole milk medium due to the formation of ferric sulphide. As a routine procedure these tests are but rarely undertaken in the examination of ordinary water samples, though they may be of considerable utility in the case of sewage effluents.

The Bacteriology of Sewage.

The methods employed for the bacteriological examination of sewage are identical with those used for potable water, except that in the case of sewage it is necessary to carry out much higher dilution of test quantities. The search for organisms of the *coli-aerogenes* group, for fæcal streptococci and for anaerobes, is essentially the same in both cases. In general it is enough to obtain a gelatin count at 20° C., an agar count at 37° C., and to search for colon bacteria. The examination of crude sewage is usually necessary only where the result is to be used as a control on the examination of effluents—a comparison which is frequently resorted to in estimating the efficiency of a disposal plant. Although the data so obtained are of great practical importance as furnishing evidence of the various methods of purification commonly in use, they are perhaps best discussed and dealt with in textbooks specifically devoted to the subject.

There is, however, a series of bacteriological problems of great practical import wrapt up in the succession of changes by which sewage is purified. All the methods of sewage disposal at present in use are conducted on principles that are purely empirical. Moreover, the changes which sewage undergoes have to be interpreted chemically in terms of oxidation and reduction, while in respect of the bacterial agents which induce these changes there is unfortunately little reliable evidence to offer.

The organisms to be found in fresh sewage are primarily those of the intestinal tract. In their new habitat they meet with a rich and varied food supply, but their environment as regards temperature and distribution is completely altered. The earliest fermentation in sewage consists in the decomposition of urea to form ammonia, a change that takes place with such ease and rapidity that it is generally impossible to detect urea when the sewage arrives at a disposal plant. A large number of organisms which would appear to the bacteriologist as being of widely different characters have in common the power to effect this decomposition. Broadly speaking, the bacteria which are responsible for the purification of sewage are referable to two main groups—the anaerobic and the aerobic. It may be assumed that the former are responsible for the hydrolysis of protein and cellulose, the saponification of fats, the reduction of nitrates and sulphates, and the decomposition of urea. Of the liquefaction of protein as it occurs in the process of sewage purification we know next to nothing from the bacteriological standpoint. The reaction results in the formation of albumoses, peptones, amino-acids and amines, as in digestion, with a further decomposition to ammonia, carbon dioxide, methane and hydrogen. Cellulose, it may be inferred, is converted into pectins, starches and sugars, which are further decomposed to yield organic acids, with carbon dioxide and methane as by-products. Another important effect which is attributed to anaerobes consists in the saponification of fats, but here too the details of the bacterial part of the process are still obscure. In certain samples which are slightly acid fatty material may be abundant, and it would seem that proteolytic changes resulting in the formation of alkaline products may be a necessary preliminary to saponification and thus to emulsification. Anaerobic action in a sewage may be said, therefore, to result in the reduction of solid materials into simpler liquid and gaseous constituents.

On the other hand the aerobic bacteria are responsible for oxidative changes in the contents of sewage. The state of ‘ nitrification ’ by which ammonia is converted first into nitrite and then into nitrate is to be taken as only one of the processes of oxidation. Although the processes of ‘ nitrification ’ and ‘ denitrification ’ have been extensively studied in the past both by chemist and bacteriologist alike it may be said they still baffle analysis.

There are, however, certain directions in which valuable knowledge of sewage purification has been obtained in recent years. In studying the rate of deoxygenation of polluted waters, Theriault (1926) has found that, in the oxygen-demand curve he has plotted out, there are two periods separated by a short lag. The first period, which occupies about 10 days, is characterized by the production of carbon dioxide, while the second period, which may last for months, and during which there is a greatly increased oxygen demand, is the stage of nitrification. With these findings may be correlated the results of recent investigations dealing with the disposal of sludge. Mohlman (1928) quotes researches on this problem published in Russia, Germany and America. The initial stage in sludge digestion consists in a fermentation of carbohydrates and the production of acidity which may be sufficient to inhibit or retard proteolysis. By the addition of lime it is possible to cut short this initial acid stage, and so accelerate the digestion of proteins and fats. There would appear to be considerable resemblance between these results and the known facts in respect of the behaviour in culture media of the better known saccharolytic and proteolytic anaerobes.

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CHAPTER III. BACTERIOLOGICAL ASPECTS OF THE DAIRY INDUSTRY.

BY R. L. VOLLUM (OXFORD).

THE methods employed in the practice of dairying are based on the thesis that it is essentially a fermentation industry, so that, in the various processes connected with it, success depends pre-eminently on the control of micro-organisms. In the production of milk or cream for use as such, the bacterial flora is all important. In the manufacture of butter, the flavour and keeping qualities which establish its commercial value are dependent on the proper control of bacterial activity. The control of the processes involved in the manufacture of cheese depend on the application of bacteriological data. Throughout every step in every process from the milking of the cow until the dairy product reaches its final consumer, the fundamental necessity is control of the bacterial flora.

The Bacterial Flora of Milk.

Milk as secreted in the normal udder of the healthy cow is sterile, but it is now well established that organisms may enter the udder *via* the teat canal, and that a limited flora may establish itself in the udder, and persist without necessarily giving rise to any symptoms of inflammation. Thus milk, even when drawn with the strictest aseptic precautions, contains variable numbers and types of bacteria. The flora of the fore-milk drawn at the beginning and in the early stages of milking is particularly inclined to be variable, whereas in the strippings, or milk drawn during the later stages of milking the bacterial content is usually low, more constant, and of well-defined types. These consist largely of micrococci, some of which appear to be identical with the common pyogenic staphylococci. There are also a few types of streptococci which differ from the common milk-souring organism *Streptococcus lactis*, and are soon overgrown by the latter in milk (Jones, 1921).

Some strains of the organisms which have been isolated from the udder have a specific action on milk, but the causative organisms of the more important fermentations of milk are chiefly those which obtain entrance during and subsequent to the operations of milking. It would appear, therefore, that the bacterial contamination of milk is largely preventable, and it necessarily follows that the spoiling or fermentation of milk is preventable or controllable just in so far as the dairyman is successful in preventing and controlling the entrance and multiplication of organisms.

From the variety and number of possible sources of contamination of milk, it naturally follows that an infinite variety of organisms may be found in milk under certain circumstances. The air of the milking shed, dust from fodder and bedding, pasture, water supply, utensils, cow dung, the skin and hair of the cow, and the milker himself may all contribute a quota to the bacterial flora. However, certain main types are found to recur with considerable regularity in the average milk, and under normal conditions these will predominate over the others, except in the case of some specific milk defects due to the growth of some particular organism.

The most important organisms causing normal fermentations of milk and milk products are those which produce lactic acid. These have been investigated by numerous workers, but chiefly by Orla-Jensen (1919), who has classified them in the following genera:

Streptococcus. Forms dextro lactic acid and grows well in milk.

Thermobacterium. Long rods. Grows at 22 to 50° C. Forms lævo or inactive lactic acid. Rapidly breaks down casein.

Streptobacterium. Occurs in chains. Optimum temp. 30° C. Forms inactive or dextro lactic acid. Usually grows well in milk.

Betabacterium. Grows poorly in milk, but thrives in presence of calcium lactate. Usually does not attack casein. Forms inactive lactic acid.

The lactic acid bacteria usually produce a much higher concentration of acid in milk than the streptococci, and they begin to develop in milk only after the streptococci have brought about souring and have begun to decrease in numbers. These organisms are of considerable importance in the process of cheese ripening, and in the fermentation of certain milk beverages.

Streptococcus lactis. This is the organism which predominates in normal sour milk. It is known in the literature under the following synonyms: *Bact. lactis* Lister, *Strep. acidi lactici* Grotenfeld, *Bact. lactis acidi* Leichmann, *Bact. guntheri* Lehmann and Neumann, and *Strep. lacticus* Kruse.

It is a Gram-positive, oval-shaped diplococcus. Grown in milk, it appears in pairs and short chains. Its optimum temperature is 30° C., but it grows readily at temperatures from 15 to 37° C. Pure cultures in milk ferment lactose to lactic acid and produce a homogeneous gelatinous clot which has a pleasant acid flavour. According to Orla-Jensen (1919), it occurs in cow dung. It may, therefore, be distributed over fields and pastures and gain entrance to the milk not only by direct contamination with dung, but also by dust from bedding and fodder, and from the skin of the cow. *Strep. lactis* invariably occurs in milk under normal conditions.

Several other streptococci of similar type are frequently found in milk, and have in common with *Strep. lactis* the property of fermenting lactose to lactic acid. *Strep. cremoris* produces certain aromatic substances as well as lactic acid. It occurs in sour cream, and is frequently used in 'starters' or cultures to sour cream for butter making and to impart flavour to the butter. At temperatures of 10to 18° C. it forms capsules, and may turn milk ropy.

Strep. thermophilus grows best at 40 to 45° C., and is found in milk which has been kept at a high temperature.

Strep. bovis, which occurs in cow dung, ferments lactose and attacks casein.

Thermobacterium. These organisms can withstand temperatures of 75° C., and their optimum temperature for growth is 40 to 45° C. They are usually large, Gram-positive rods but may show Gram-negative forms and granular staining. They are micro-aerophilic. They grow best in media containing casein as a source of nitrogen. At times they may form capsules, and produce ropiness in milk. *B. bulgaricus* is of this type.

Streptobacterium. As their name implies, these organisms tend to grow in chains. They will grow in the presence of oxygen, and their optimum temperature is about 30° C. In other respects they resemble the thermobacteria. *Streptobacterium casei,* which plays an important part in cheese ripening, belongs to this group.

Betabacterium. Organisms of this type grow best at temperatures of 20 to 30° C. They do not grow well in milk in pure culture, as they cannot attack casein. *B. casei* γ and *B. casei* δ, which belong to this group, proliferate in cheese during the ripening stages, using the calcium lactate as a source of carbon. *Betabacterium caucasicum,* which is found in Kefir grains in association with a yeast, is also of this type. The betabacteria produce a certain amount of gas and succinic acid as well as lactic acid.

Colon-aerogenes group. In addition to the typical lactose-fermenting milk organisms mentioned above, milk is almost invariably contaminated to a certain extent by organisms of the *colon-aerogenes* group, particularly *Bact. coli*, *Bact. aerogenes* and *Bact. acidi-lactici*, which ferment lactose, producing acid and gas. According to Rogers (1921), *Bact. aerogenes* is common in soil, and in substances contaminated with soil, while *Bact. coli* is found only in connection with recent fæcal contamination. Both of these types may be introduced into milk, and their presence in large numbers in freshly drawn milk may be taken as an indication of extensive fæcal pollution. In raw milk, drawn with normal care and kept cool, it should not be possible to detect these organisms in test quantities of 0·1 c.cm. Because of their ability to grow rapidly in milk, and produce gas and disagreeable odours and tastes, they form one of the most serious problems with which the dairyman has to contend.

Anaerobes. Of the anaerobes which occur in milk the most frequent are *B. welchii* and *B. sporogenes* (vide Chap. IX). The presence of the former can be readily detected by inoculating graduated quantities of the test milk into sterilized whole milk covered with vaseline or liquid paraffin. The tubes of the medium with their inocula are heated to 80° C. for 20 minutes, and then incubated at 38° C. The occurrence of the characteristic stormy fermentation in 12 to 24 hours—a reaction which is given by no other organism in milk—is diagnostic of *B. welchii*. *B. sporogenes*, which is less frequently found, produces in milk a soft, friable clot which is rapidly digested, so that there ultimately results a straw-coloured, clear whey with fine, granular deposit.

Small numbers of numerous other organisms may be present in normal milk, but as they are usually suppressed by the growth of the types mentioned above, they are of less importance. Spore-forming bacteria from the soil, alkali-forming bacteria, *Proteus vulgaris*, and fluorescent bacteria from water may all be present. A few yeasts and moulds are usually present as well.

SOURING OF MILK.

The bacterial flora of the milk and the equilibrium of the different types depends not only on the initial contamination, but also on the age of the milk, and above all, on the temperature at which it has been kept. The organisms which tend to predominate at different temperatures are as follows:

0°– 5° C.	The fluorescent bacteria.
5°–10° C.	Fluorescent bacteria, *B. proteus*, micrococci and alkali-forming bacteria.
10°–15° C.	Streptococci and some species of *Bact. aerogenes*.
15°–30° C.	Streptococci, especially *Strep. lactis*.
30°–40° C.	*Bact. coli*, *Bact. aerogenes*, the lactic-acid-forming bacteria and a few streptococci.
40°–50° C.	Lactic-acid-forming bacteria, saccharomycetes, *Strep. thermophilus* and *Strep. fæcium*.

In milk kept at low temperatures the bacterial development is very slight, but certain proteolytic organisms may develop, and if the initial contamination with these types is high, undesirable changes will occur.

In milk kept at ordinary room temperature, organisms will develop fairly rapidly and a well-defined series of changes will take place corresponding with the sequence in the predominating bacterial types. At first most of the organisms present develop fairly rapidly, and the milk acquires an unpleasant, stale taste. The lactic-acid-forming streptococci then gradually begin to predominate, as the lactic acid formed suppresses the multiplication of other types of organisms. The stale flavour is then masked by the definite sour taste. If the milk is heavily contaminated with organisms of the *colon-aerogenes* group, they will continue to grow, and produce gas, and the clot formed will be broken up by the gas bubbles. If the contamination with this group is minimal, they may be suppressed by the streptococci, and the clot formed will be homogeneous. The streptococci will not be able to survive in the presence of the acid they have produced, and they will gradually give way to the lactic acid bacteria. As these develop, the acidity will increase from about 0·6 to more than 1 per cent. The acid medium thus formed is favourable for the development of yeasts and moulds, which will grow readily, utilizing part of the acid as a source of energy and neutralizing the remainder by ammonia and the products of protein hydrolysis. When all the lactose is fermented, and all the acid has disappeared, the surviving putrefactive organisms begin to flourish and the casein will be destroyed.

DEFECTS OF MILK.

The more common defects of milk due to bacterial activity are as follows:

Fermenting and gassy milk. These defects, characterized by the evolution of gas before or after coagulation, are due to the fermentation of lactose by gas-producing organisms. They are generally caused by bacteria of the *colon-aerogenes* group, but may in some cases be due to yeast, or to *B. welchii.*

Ropy or slimy milk. Milk suffering from this defect may be drawn out into threads sometimes over a yard in length. It is most likely to develop in milk kept for some time at 15 to 20° C., and is due to the proliferation of capsulated organisms. Certain of the lactic-acid-producing organisms—particularly *Strep. cremoris*—may produce ropiness in milk at low temperatures. Other organisms which may be responsible for this condition are *Micrococcus freudenreichii* Guillebeau (1891), *Micrococcus lactis viscosus B* (Conn, Esten and Stocking, 1906), and *B. lactis viscosus* Adametz (1891). Ropiness in milk is also frequently caused by certain slime-producing organisms present in water. Buchanan and Hammer (1915) have described 33 species of bacteria which may cause sliminess or ropiness in milk.

Premature coagulation. This defect is usually due to the exceptionally active development of peptonizing lactic acid bacteria, such as *Strep. liquefaciens.* The coagulation is probably due to an enzyme, as the milk is not appreciably acid at the time of clotting.

Coloured milk. This defect is due to the growth of chromogenic organisms. Milk is turned blue by *B. syncyaneus*; red by *B. erythrogenes*; and yellow by *B. synxantum.* *B. prodigiosus* may produce red spots on the surface. *B. rudensis* has been found to produce red spots in cheese. These defects usually develop only when milk is left to stand at low temperature. They are not common.

Unclean, sour taste. This taste is not due to any specific organism, but it is usually caused by the rapid development of the bacterial flora other than the lactic-acid-forming types. It can usually be avoided by efficient cooling of the milk.

Turnip taste. This taste may be present in the milk when the cows have been fed on turnips, but it may also be due to *Bact. coli* or *B. liquefaciens fluorescens.* Some species of mould are also said to produce this flavour.

Soapy taste. This taste may be produced by alkali-producing organisms such as *B. sapolacticum* in milk kept at low temperatures.

Bitter taste. This defect is frequently due to products of protein hydrolysis produced by peptonizing bacteria. Harrison (1902) has found a yeast, *Torula amara*, responsible for an outbreak of bitter milk in Ontario. Sadler (1911) isolated an atypical strain of *Strep. lactis* which produced a bitter taste and an aroma of caramel in milk. Butter and cheese made from infected milk acquired the same taint. This taint has frequently been found in milk and dairy products, and is variously described as a burnt flavour, malty flavour, and condensed-milk flavour. It seems fairly widespread, and has been recorded by various workers in Europe, England and America. A review of the literature on the subject is given by Sadler (1926), who records a recent serious outbreak of this defect. The atypical *Strep. lactis* was again isolated in this case.

PATHOGENIC ORGANISMS IN MILK.

The defects of milk mentioned above are more or less obvious, and can be detected easily by sight, smell or taste. A more serious danger is contamination with organisms pathogenic for the consumer, which give no immediate indication of their presence. Milk and milk products may be the vehicle of infection not only for man, but also for calves, pigs and other farm animals which are frequently fed on skim milk and whey. Pathogenic organisms may be derived from diseased cows—the milk being already contaminated within the udder. Any of the organisms pathogenic for man may be introduced into the milk through handling by infected persons. Provided the organism can survive—it need not necessarily grow—the milk may be a source of infection.

Pathogenic Organisms of Bovine Origin.

Mastitis. The milk of cows suffering from mastitis may contain large numbers of *Strep. mastitidis,* and must be regarded as undesirable for human consumption. If the mastitis is severe, the milk may contain pus and blood in appreciable amounts.

Tuberculosis. In tuberculosis of the udder, contamination of the milk is unavoidable. If other organs are infected, the excreta are likely to contain tubercle bacilli, and since milk is almost invariably polluted to a certain extent with dung, it is likely to contain *B. tuberculosis* whether the udder is infected or not. Furthermore, it has been shown by Griffith (1913) that tuberculous cows excrete tubercle bacilli in the milk in the absence of udder infection. *B. tuberculosis* does not proliferate in milk, but it can live and retain its virulence in milk and milk products for more than two months (Mohler, Washburn and Rogers, 1909).

Foot-and-mouth disease. Cows infected with foot-and-mouth disease may have vesicles on the udder and teats, and the milk may be contaminated with the virus.

Contagious abortion. Cows suffering from contagious abortion may excrete large numbers of *B. abortus* in their milk. This organism is probably pathogenic to man and it may infect many of the common farm animals. A very similar organism, *B. melitensis,* which causes Malta fever in man, is to be found in the milk of goats suffering from this disease. (Vide Vol. V.)

The question of the pathogenicity for man of streptococci and tubercle bacilli of bovine origin, and of the virus of foot-and-mouth disease, has been discussed at great length in the literature, and need not be dealt with here. Reviews of the literature and records of cases are given by Swithinbank and Newman (1903), Lane-Claypon (1916) and Rosenau (1927).

Pathogenic Organisms of Human Origin.

Milk may be contaminated in numerous ways by human carriers of pathogenic organisms; by hands contaminated with excreta; by droplets expelled in coughing or sneezing and by saliva used to moisten the hands in that abominable practice of wet milking. There are numerous records of milk-borne epidemics of typhoid fever, scarlet fever, diphtheria, septic sore throat, and diarrhœa (Swithinbank and Newman, 1903; Lane-Claypon, 1916). Milk-borne epidemics of infantile paralysis and epidemic arthritic erythema are also recorded by Rosenau (1927).

BACTERICIDAL PROPERTIES OF MILK.

It has been found by numerous workers that the number of bacteria in freshly drawn milk tends to decrease during the first few hours, when estimated by the total plate count. The apparent bactericidal power is active for a short time at 37° C., while at 15° C. the action is less marked but more prolonged (Rosenau and McCoy, 1909). The action appears to be specific for certain organisms in each sample of milk, but the milk of different cows affects different organisms (Chambers, 1920). The apparent bactericidal action is destroyed by heat. A part of the reduction is due to agglutination of the bacteria, which is not detected by the methods of plating out. Part may also be due to the antagonistic action of different species of organisms, and it is possible that part may be due to the leucocytes in the milk. It is also possible that certain bactericidal substances may be derived from the blood, and be secreted in the milk, but their importance from the point of view of the bacteriology of milk is negligible. The literature on the subject has been reviewed by Lane-Claypon (1916).

The Control of the Bacterial Flora of Milk Supplies.

The ideal to be aimed at in dairy practice is the production of a clean milk from healthy cows in hygienic surroundings, and the proper management of the milk by clean, healthy workers who are competent to appreciate all those factors which may influence the product in any way. Successful accomplishment in any dairying enterprise is dependent on cleanliness, which in turn is dependent on the appreciation and application of hygienic principles. These facts were recognized long before the development of the science of bacteriology by Twamley (1787) who advises, '. . . to take care the Utensils it is set up in are exceeding clean'; and remarks that 'Nothing is more commendable in a Dairymaid than cleanliness, nor will anything cause them to be more esteemed'.

The investigations of North (1918), Delépine (1918), Freear, Buckley and Williams (1919), Ayers, Cook and Clemmer (1918), Sadler, Kelly and Martin (1924), and others have demonstrated that it is possible to produce and deliver to the consumer, under commercial conditions, raw milk, untreated by heat or chemicals, containing less than 10,000 organisms per cubic centimetre. The attainment of this standard naturally involves that the utmost cleanliness shall be observed in all processes in the production of the milk.

It has been shown by numerous investigators in recent years, and particularly by Prucha (1920) and his co-workers, that unclean utensils are probably the source of more contamination in milk than all other factors combined. It is impossible to lay too much stress on the necessity for cleaning and sterilizing all milking pails, churns, covers, strainers, separators and other utensils used in the dairy. A simple quantitative bacteriological examination will frequently show that an apparently clean pail or churn, which has been insufficiently sterilized and allowed to stand 12 hours, contains enough organisms to contribute from 10,000 to 500,000 organisms to each cubic centimetre of milk the vessel would hold.

Heavy contamination from unclean utensils or other sources will, of course, impair the quality of the milk, and reduce its period of usability. There is, however, the more serious danger that even clean milk may be contaminated with pathogenic organisms from the cow, or from an undetected carrier among the dairy workers. In order to eliminate this danger, it is desirable that milk should be subjected to some treatment which will certainly destroy any pathogenic organisms and make it safe for consumption. The methods of treating milk which have been practised for the purpose of destroying pathogenic micro-organisms are: the addition of antiseptics ; subjecting milk to the action of an electric current ; and most important of all, the application of heat in the process of pasteurization.

Antiseptics.

Antiseptics such as formaldehyde, boric acid, salicylic acid, and hydrogen peroxide added to milk in a suitable concentration may kill a certain number of organisms—including the pathogenic types—and prevent the development of others. These substances, however, are to a certain extent poisonous and their use is now generally prohibited. The indiscriminate use of antiseptic substances may tend to encourage a disregard for cleanliness.

Electricity.

A method has been described by Beattie and Lewis (1920), who claim that by it milk can be rendered free from *Bact. coli* and *B. tuberculosis*. The temperature effect is of very short duration, and is apparently not the principal factor in the destruction of the bacteria.

Pasteurization.

The most commonly used method is the application of heat in the process of pasteurization. Its chief modifications are the 'flash' and 'holder' processes.

In the flash process, the milk is momentarily heated to a temperature of 70° to 90° C., and subsequently cooled. The apparatus used for this process does not insure that every particle of the milk reaches the maximum temperature, and the method is therefore neither efficient nor safe. It has the additional disadvantage that it may impart a definite cooked flavour to the milk.

The 'holder' process, now used in all modern pasteurizing plants, is more efficient and satisfactory. Briefly, the process consists in quickly raising the temperature of the milk to 61 to 63° C., holding it at that temperature for 30 minutes, and subsequently cooling it. By this treatment all pathogenic organisms, except those which form spores, will be killed. The organisms of the *colon-aerogenes* group, many of the micrococci, and the greater proportion of the *Streptococcus lactis* type will also be destroyed. An appreciable number of the true lactic-acid-forming organisms, both streptococci and bacteria, will survive this treatment. Therefore, milk properly pasteurized and not subsequently recontaminated will still sour normally by the formation of lactic acid, if left at a temperature suitable for the development of the lactic-acid-forming organisms. If the pasteurizing temperature has been too high, or has been maintained for such a time that all of the lactic-acid-forming organisms are destroyed, the spore-bearing bacteria will naturally predominate and the milk will putrefy instead of souring normally.

The pasteurizing apparatus should comply with certain definite specifications, and the process should be carried out by competent operators under proper scientific control. Regular bacteriological examinations of the milk should be made to detect any possible faults in the apparatus or in its operation. The essential details of the mechanical features and the operation of efficient pasteurizing plants, and the analytical methods for controlling such plants, have been described by the American Public Health Association (1920).

The object of pasteurization of milk is the destruction of any pathogenic organisms it may contain. The ideal test of the efficiency of the process would, therefore, involve the demonstration of the presence or absence of pathogenic bacteria in the pasteurized milk. A direct test of this nature presents almost insuperable difficulties, but an indication of the probable effect of the pasteurizing process on pathogenic organisms may be obtained by quantitative bacteriological analyses of the milk before and after pasteurization.

The determination of the total number of viable organisms in the milk before and after pasteurization, and the expression of the results in terms of percentage reduction is a common practice. There are certain inherent fallacies in considering the percentage reduction arrived at by this method as percentage efficiency. The different types of organisms found in milk vary considerably in their resistance to heat. The percentage reduction will depend to some extent, therefore, on the equilibrium of the bacterial flora at the time of pasteurization. The predominating type at that time will depend not only on the initial contamination, but on the age of the milk, the temperature at which it has been kept and other factors which may influence the equilibrium of the bacterial flora. Milk with an initial high bacterial content may show a reduction of more than 99·5 per cent. and still contain an undesirably large number of viable organisms ; while clean milk with a low bacterial content may show a much lower percentage reduction when subjected to the same treatment. It is, therefore, impossible to set up any general standard of efficiency in terms of percentage reduction. In the interpretation of results it is essential in all cases to take into consideration the history of the milk and the total number of organisms before and after pasteurization. The percentage-reduction figure alone is of very little value.

Information of considerable value about the efficiency of pasteurization may be gained by the quantitative determination of the organisms of the *colon-aerogenes* group present in the milk before and after pasteurization. Bacteria of this type are almost invariably present even in normally clean raw milk. The effect of the pasteurizing process on organisms of this type is more uniform than on the bacterial flora as a whole, and is likely to approximate more closely to the effect on the pathogenic organisms which may be found in milk. It is improbable that such pathogenic organisms will survive conditions under which all the organisms of the *colon-aerogenes* group are killed. If the milk is properly pasteurized it should not be possible to detect the presence of organisms of this group, which ferment lactose to acid and gas, when quantities of 1 c.cm. are used for the test.

Milk Standards and the Grading of Milk.

There is no universally accepted standard of quality, but various grades are usually defined by the local public health authorities. Throughout the greater part of Canada and the United States the highest grade of milk is known as ‘ Certified Milk ’. This milk is produced under the supervision and direction of a medical milk commission working under clearly defined rules. Milk produced under less carefully controlled conditions is frequently graded as A, B or C, according to the hygienic conditions under which it is produced, and according to its bacterial content. The standards of bacterial content frequently adopted for these grades are as follows :

Grade A, raw. The bacterial count should not be more than 10,000 per c.cm.

Grade A, pasteurized. The bacterial count should not be more than 200,000 before pasteurization, and not more than 10,000 per c.cm. when delivered to the consumer.

Grade B. The bacterial count should not be more than 1,000,000 per c.cm. before pasteurization, and not more than 50,000 at the time of delivery.

Grade C. The bacterial count is more than 1,000,000 per c.cm. It must be pasteurized and the bacterial count must not be more than 50,000 at the time of delivery. The sale of milk of this grade is frequently prohibited.

All milk containing more than 10,000 bacteria per c.cm. should be pasteurized, but never more than once.

Bacteriological Examination of Milk.

The grading of milk naturally involves some method of bacteriological examination. The procedure to be adopted depends on the information required and on the laboratory facilities available. Methods suitable for routine examination of milk have been described by the American Public Health Association (1921). The determination of any particular type of organism will naturally require a special technique. Useful information about the bacterial flora of milk may be obtained by the simple fermentation test, or the methylene-blue reductase test. The grading of milk may be based on one or more of these tests, according to the standards established.

Collection of samples. The can or bottle should be shaken until the milk is thoroughly mixed. The sample is removed with a sterile tube, long enough to reach to the bottom of the vessel. The sample should be kept in stoppered bottles, on ice, until examination. It should be examined as soon as possible.

Determination of total count. The milk is diluted with sterile water in the proportion of 1 : 100, 1 : 1,000 and 1 : 10,000. The most suitable dilution for any particular milk will depend on the quality. In the case of unknown milk it is safer to plate all dilutions. One c.cm. of the dilution is pipetted into a sterile petri dish. 10 to 15 c.cm. of nutrient agar are poured into the plate, and mixed with the milk by a gentle rotary motion. The plates should be made in duplicate. After 48 hours incubation at 37·5° C., the colonies are counted, and the number of colonies per c.cm. calculated. In the interpretation of results it is necessary to bear in mind that the colonies do not necessarily develop from single organisms, but usually from small groups, such as chains of streptococci.

Ayers and Mudge (1920) have suggested the use of agar media containing skim milk powder as the chief ingredient. With this medium the total count is considerably larger, and the various types of organisms can be differentiated. The colonies are counted as follows:

1. Total number of colonies.
2. Vigorous lactic acid bacteria. These colonies are surrounded by a turbid zone, or have an indistinct edge.
3. Weak lactic acid organisms. The plate is washed over with an indicator such as brom-cresol-purple, to distinguish the

acid-forming colonies. The number of vigorous acid-forming colonies is subtracted from the total.

4. Peptonizing organisms. The plate is treated with a 5 per cent. solution of acetic acid which precipitates the dissolved casein of the milk powder except around the peptonizing colonies. These show a clear zone.
5. Alkali-forming and indifferent organisms. The number of acid-forming colonies is added to the number of peptonizing colonies, and this figure is subtracted from the total number of colonies. The difference represents the number of alkali-forming and indifferent colonies.

By using an agar medium containing 1 per cent. lactose, and an indicator such as brom-thymol-blue or brom-cresol-purple, the total number of colonies, and the number of acid-forming colonies can be determined.

Direct Microscopic Count. (*Breed method.*)

In this method the number of organisms in the milk is determined by direct microscopic examination. The technique of Breed and Brew (1917) is as follows:

With a capillary pipette calibrated to deliver 0·1 c.cm., this quantity of milk is placed on a microscopic slide and evenly spread over an area of 1 sq. cm. The slide is dried on a warm plate within 5 to 10 minutes, dipped in xylol to remove the fat, fixed in alcohol, and then stained with methylene blue. After washing in water, it is decolourized in alcohol until the background is a faint blue. The film is examined microscopically and the organisms in several fields are counted. To facilitate calculation, an eye-piece micrometer is usually used, and the microscope so adjusted that the area of each field bears some simple ratio to a square centimetre. This method is simple, fairly rapid, and requires much less apparatus and equipment than the plating method. The slides may be kept as permanent records.

By the Breed method it is possible to determine the number of individual organisms, as well as the number of clumps of bacteria in the milk. For this reason, and because some of the organisms may not grow on agar, the direct count is, on the average, about five times as high as the plate count of fresh raw milk. It is, of course, impossible to distinguish between dead and living organisms in the milk by this method, but the total number of organisms which have developed can be determined. Grossly contaminated milk which has been pasteurized may appear fairly good when examined by the plate method, but the excessive bacterial development will be detected by the direct method. By the use of both methods, considerable information about the milk may be obtained. A valuable feature of the Breed method is that it enables one to determine readily the presence of an excessive number of long chained streptococci and leucocytes, which indicate mastitis in the cow. The differentiation of organisms on morphology alone is of course unreliable, but nevertheless the experienced bacteriologist will be able to recognize certain types commonly found in milk, such as *Strep. lactis*, and may be able to detect a grossly abnormal flora.

Determination of colon-aerogenes *Group.*

The presence of organisms of the *colon-aerogenes* group, which ferment lactose to acid and gas, may be used as an index of fæcal pollution in raw milk. It may also be used in determining the efficiency of pasteurization. In the interpretation of results it is necessary to take into consideration the age of the milk, the temperature at which it has been held, and other factors which might influence the growth of organisms of this type. In clean raw milk, properly handled, it should not be possible to detect *Bact. coli* in test quantities of 0·1 c.cm.

Fermentation Test.

The fermentation test (Orla-Jensen, 1921) is of considerable value in judging the suitability of milk for cheese making, and also in the control and grading of ordinary market milk.

Forty c.cm. of the milk is measured into a large sterile test-tube, about 2 cm. in diameter, and the tube placed in a water bath at 37·5° C. The time required for clotting and the type of clot formed gives an indication of the number and type of organisms present. Good fresh milk should remain liquid for at least 12 hours, and a high-grade clean milk may remain liquid for 24 hours or longer. Clean milk, in which the *Strep. lactis* type predominates, forms a homogeneous, gelatinous clot, without exudation of whey, and without gas bubbles. Slight contamination with *Bact. coli* will produce slight cracks or bubbles in the clot, or streaks up the side of the tube. If heavily contaminated with *Bact. coli*, a ‘ gassy ’ or ‘ stormy ’ clot will be formed. The clot is broken up by gas bubbles and whey is exuded. If the milk contains relatively few true lactic acid organisms, and a number of gas-producing bacteria, the clot may be ‘ spongy ’, due to the rapid evolution of gas before and during coagulation. A ‘ cheesy ’ type of fermentation, where the clot is greatly contracted and the clear whey well separated, may be produced by organisms which secrete enzymes similar to rennet, such as the peptonizing lactic acid streptococci. Very rapid and almost complete peptonization or dissolving of the casein has been recorded by Sadler and Mounce (1922), who found a staphylococcus to be the most common cause of this type of reaction.

Many gradations of these various types of clot may be found. The chief value of the test is in detecting the presence of gas-forming organisms in undue numbers. This type is favoured by the temperature at which the test is conducted. The relative numbers of bacteria in different samples of milk may be roughly estimated from the time required for clotting.

Reductase Test.

The reductase test, devised by Barthel and Orla-Jensen, depends on the fact that living cells, including micro-organisms, have reducing properties, which may be shown by their behaviour towards methylene-blue. Forty c.cm. of milk are mixed with 1 c.cm. of a 0·5 per cent. solution of methylene-blue in a large test-tube, and incubated at 37·5° C. as in the fermentation test. The time required for the decolourization is noted.

Many factors enter into the reduction of the methylene-blue. The milk itself and the leucocytes in it have a reducing action. The different types of organisms present in the milk vary in their reducing powers, and each type will vary according to its rate of growth. In spite of these varying factors, the reductase test has been found very useful in grading milk, and in determining roughly the number of organisms present. Barthel and Orla-Jensen (Orla-Jensen, 1921) have suggested the grading of milk and cream into four classes as follows:

Class 1. Good milk, not decolourized in 5½ hours, containing less than 500,000 bacteria per c.cm.
Class 2. Fair milk, decolourized in 2 to 5½ hours, containing 500,000 to 4,000,000 bacteria per c.cm.
Class 3. Bad milk, decolourized in 20 minutes to 2 hours, containing 4,000,000 to 20,000,000 bacteria per c.cm.
Class 4. Very bad milk, decolourized in 20 minutes or less, containing over 20,000,000 bacteria per c.cm.

Detection of B. tuberculosis *in Milk.*

B. tuberculosis is probably found in milk more frequently than any other pathogenic organism. The ordinary method for detecting this organism by microscopical examination is to centrifuge 50 c.cms. of the milk, make smears of the deposit, stain by the Ziehl-Nielsen method, and examine for acid-fast bacilli. If the number of tubercle bacilli present is small, they may easily be missed; and if there is a large amount of tissue debris it is difficult to make satisfactory preparations. A method for overcoming these difficulties, and for concentrating the tubercle bacilli in milk, has been described by Douglas and Meanwell (1925). The milk is digested with trypsin, then shaken with ether and the emulsion centrifuged in a closed tube. Three layers separate out—the ether containing the dissolved fat at the surface, a clear fluid below, and between the two a gelatinous layer which contains all the acid-fast bacilli. Since acid-fast bacilli other than *B. tuberculosis* may be found in milk, it is advisable to confirm the microscopical diagnosis by animal inoculation.

Butter.

Butter consists of milk fat with which is incorporated 10 to 16 per cent. of its weight of water. It also contains some casein and lactose—the amount present depending on the state of the cream before churning, and on the extent to which the butter is washed in the process of manufacture. Salt which is usually added in the proportion of 1 to 3 per cent. dissolves in the water, forming a 10 to 20 per cent. solution.

As a medium for bacterial growth, butter is not as favourable as milk or cream. The fat can only be attacked directly by fat-splitting organisms, and the growth of other organisms depends on the amount of casein and lactose present. The bacterial development is hindered to some extent by the high concentration of salt in the water.

The commercial value of butter depends on several factors—flavour, aroma, colour, moisture content, salt and texture; of these qualities, the most important are flavour and aroma, which depend very largely on the action of bacteria. These factors are found to lend themselves to very careful control.

Butter may be manufactured from sweet, fresh cream, raw or pasteurized, but the separation of the fat from the casein is easier and more complete if sour cream is used. For this reason butter is usually made from sour cream, which may be raw or pasteurized—the pasteurization taking place either before or after souring. The cream may be allowed to sour naturally, or souring may be promoted by the addition of a 'starter' culture. The introduction of 'starters' is due to Storch, in Denmark, who found that different strains of lactic-acid-producing organisms behaved differently, some producing considerable amounts of acid while others produced less acid but more aromatic substances, which were absorbed by the fat globules and imparted flavour to the butter. The 'starters' consist of cultures of these organisms in suitable proportions, grown in pasteurized milk. The organisms employed are usually varieties of *Strep. cremoris*, but other lactic-acid-producing organisms may also be used.

Cream is usually pasteurized at a higher temperature than milk, because the number of organisms present is usually very much greater. The butter fat is not appreciably affected by higher temperatures, and does not acquire a 'cooked' flavour. A temperature of 75 to 95° C., held for 15 to 20 minutes is efficient in reducing the bacterial flora, and in killing any pathogenic organisms which may be present.

Butter made from clean, sweet, fresh cream has a mild flavour—the flavour of the milk fat. Its bacterial flora will be the flora of the cream from which it is made. If due precautions as to cleanliness are observed, and if the cream is kept cool until churned and the butter kept cool until used, the flavour may remain unchanged for a short time. If the butter is not kept cool, the bacterial flora will develop. The flavour produced will depend on the types which predominate, and it is likely to be undesirable because the acidity of the butter will not be sufficient to suppress the growth of the putrefactive organisms. If the butter contains sufficient casein and lactose to favour the development of the lactic acid organisms, it will acquire a sour taste.

In modern dairy practice, butter is usually made at a central creamery from sour cream, which may be several days old. The cream has a high bacterial content—usually several hundred millions per cubic centimetre—and a high acidity. Butter made directly from such cream has a high bacterial content and a flavour which depends largely on the bacterial flora of the cream. If organisms other than the lactic-acid-forming type have grown to any extent the flavour may be unpleasant. In any case, the butter has very poor keeping qualities.

In order to overcome these difficulties, and to produce a more standard product, certain procedures are adopted. The acidity of the cream is neutralized with lime and the cream is pasteurized to destroy its heterogeneous flora. If necessary, the cream may be aerated to remove unpleasant volatile products of bacterial growth. The cream is then inoculated with a culture of lactic acid organisms known to produce a desirable flavour and aroma. The development of this ' starter ' culture is allowed to proceed until the desirable acidity is again reached, and the cream is churned. Butter thus made has a preponderance of lactic acid organisms, and these tend to inhibit the growth of other organisms which may be present. The lactic acid organisms die off after a few weeks, and undesirable bacteria which may be present can then develop. However, butter made from cream treated in this way has much better keeping qualities than butter made from raw unpasteurized cream, which is heavily contaminated with organisms likely to lead to the production of undesirable flavours. Furthermore, it is possible, by using the same 'starter' culture, to obtain regularly the same flavour and thus a more uniform product. The pasteurization of the cream and the subsequent use of good cultures have been the chief factors in making Danish butter a uniform and well-keeping product.

A practice which has gained favour in recent years is the manufacture of butter from cream which has been pasteurized after ' ripening ' or souring. The ripening may be promoted by the use of a ' starter ' culture which contains both the desirable lactic-acid-producing and aroma-producing organisms. The cream is then pasteurized, and provided that proper care is used in the processes of churning, washing and working, the butter will have a very low bacterial content, and consequently better keeping qualities.

The organisms used in the pure culture starters mentioned above are isolated from clean cream which has been allowed to sour. These organisms occur naturally in cream, and quickly predominate in clean cream allowed to sour spontaneously, producing the desirable flavour and aroma. It is therefore unnecessary to add 'starter' to a clean cream which has been produced and handled with proper hygienic precautions. Butter made from such cream has better keeping qualities and better flavour than butter made from cream heavily contaminated with undesirable organisms, even if these are killed by pasteurization.

For these reasons, of recent years, particularly in New Zealand and in Western Canada, where butter is made for export and must necessarily be shipped long distances, and must, therefore, have good keeping qualities, considerable attention has been paid to the grading of cream with respect to its suitability as the raw material for the manufacture of butter, and to the question of grading of butter before it is placed on the market. The highest grade of butter is now made from clean cream, spontaneously soured by its own bacterial flora, and subsequently pasteurized at 80 to 85° C. for 10 minutes—no 'starter' being added either before or after the process of pasteurization.

The introduction of these procedures, which has led to the production of a standard product of uniform high quality, is due largely to the work of Ruddick in New Zealand, and later as Dairy Commissioner for Canada, and of Professor C. P. Marker in Alberta.

BACTERIOLOGICAL EXAMINATION OF BUTTER.

The bacterial flora of butter consists of the organisms present in the cream, and of the organisms with which it is contaminated in the process of churning, washing, working, salting and packing. The bacterial flora of the surface of the butter, therefore, differs from that of the inside, not only because it is more exposed to contamination, but also because of the difference in oxygen tension which may influence the bacterial equilibrium.

For these reasons, it is difficult to obtain a representative sample of butter for bacteriological examination. Samples from different parts of the butter may be removed with sterile knives, and then examined separately, or a cylinder of the butter may be removed with a sterile 'trier', and a mixed sample made from different parts of the cylinder. The sample is melted in a waterbath, and dilutions made in sterile water at 37·5° C. When the dilutions have been made, the procedure is the same as that for the examination of milk by the total plate count, and the determination of the bacteria of the *colon-aerogenes* group. Gelatin plates may also be used, and incubated at room temperature to determine the total count and the number of liquefying organisms.

DEFECTS OF BUTTER.

Defects which develop in butter after manufacture are largely due to the activities of micro-organisms, and, therefore, scrupulous precautions must be taken in its management. All that has been said in regard to the cleaning and sterilizing of utensils applies with equal force in the manufacture of butter. It has been found that a large proportion of the organisms present in defective butter made from pasteurized cream are present as a result of recontamination at some stage in the process of manufacture after pasteurization (Lund, 1921; Sadler and Vollum, 1926). The recontamination is not only of importance in itself, but is an indication of the care with which the manufacture of the butter has been carried out. The degree of recontamination may be judged by the yeast content of the butter (Lund, 1921) or by the *Bact. coli* content as suggested by Sadler (1926).

Specific Defects of Butter.

Rancidity is due to the hydrolysis of the fat by fat-splitting enzymes of organisms such as *Oidium lactis* (Sayer, Rahn and Farrand, 1908). Orla-Jensen (1921) finds that *Oidium lactis, Penicillium glaucum, B. fluorescens liquefaciens* and *B. prodigiosus* all actively hydrolyse fat and cause rancidity.

Fishiness is almost invariably associated with high acidity in butter, and the decomposition of lecithin. Harrison (1913) found that fishiness might be due to small amounts of trimethylamine, which is produced by certain bacteria. Supplee (1919) and Cusick (1920) found that *B. icthyosmius*, using the decomposition products of lecithin as pabulum, could produce a fishy flavour in an acid butter.

Tallowiness is found to be due largely to oxidation. It may be brought about by the action of sunlight, or it may be induced by the natural oxidases in cream (Palmer and Combs, 1919). It may also be caused by enzymes secreted by certain moulds (Combs and Eckles, 1917). The enzymes are destroyed by efficient pasteurization.

Putrid flavours may be produced by the action of proteolytic organisms on the casein in the butter.

Cheese.

Cheese consists of the solid matter in milk—fat, casein and insoluble salts—together with more or less moisture containing lactose and salts in solution. The varieties of cheese are almost innumerable, and a more or less specialized technique is employed in the manufacture of each variety. Slight deviations from the established technique may bring about variations in the equilibrium of the bacterial flora and also affect other factors in such a way that the characteristics of the resulting cheese are changed to some degree. For these reasons it is impracticable to discuss in other than very general terms the bacteriology of cheese.

In general, cheese is made from raw milk, and, therefore, as in all other branches of dairying, it is highly essential that the raw material should be of good quality if the final product is to be satisfactory. It is now well established that typical, high-grade cheese of most of the well-known commercial varieties can be manufactured from pasteurized milk, subsequently inoculated with the appropriate culture to produce the desirable qualities. By this method the cheese maker can more easily control the process and eliminate certain troublesome organisms, such as the gas-producing bacteria of the *colon-aerogenes* group. At the same time, any pathogenic organisms which may be present are killed.

The manufacture of cheese may be divided into two main stages—the preparation of the cheese, and the process of ripening. In each stage bacteria play an important part. In the first stage, the curd is separated from the whey, either by the action of lactic acid alone, producing the 'acid curd' cheeses, or by the action of rennet in the presence of lactic acid, producing the 'rennet curd' cheese. In both cases the lactic acid is produced in the milk by organisms of the *Strep. lactis* type. Some cheeses are ready for use at this stage, but the majority undergo a ripening process in which bacteria, moulds, or enzymes bring about a series of changes in the texture and flavour of the curd.

Acid curd cheeses. The majority of cheeses of this type are local products, and are not of great commercial importance. They may be used fresh, but many varieties are allowed to ripen. The ripening may be brought about by the action of lactic acid organisms, in conjunction with butyric acid organisms, which impart a piquant flavour to the cheese; or in conjunction with various species of yeasts or moulds, which give the characteristic properties to the different cheeses.

Rennet curd cheeses. When first prepared, the curd of these cheeses is generally tough and rubbery in texture, insoluble, and devoid of any flavour or aroma. During the ripening or maturing process the curd undergoes a series of chemical and physical changes which may alter its texture, digestibility and solubility, and the cheese acquires a characteristic flavour and aroma. The cause of these changes and the method by which they are brought about has been the subject of extensive investigation and controversy ever since the first work on the subject by Duclaux in 1878. The voluminous literature on the subject has recently been reviewed by Hucker (1921[1]) and some details of the modern theories are given by Orla-Jensen (1921) and Barthel (1923).

The rennet curd cheeses are divided into the ‘ hard rennet ’ and the ‘ soft rennet ’ varieties. In the former, the ripening proceeds uniformly throughout the whole cheese, while in the latter the ripening agent grows on the surface of the cheese and produces enzymes which diffuse into it and bring about the characteristic changes. Cheddar and Emmenthal cheeses are examples of the hard rennet variety. The chief organisms present during the ripening stages appear to be the lactic-acid-forming rods. In the ripening of Emmenthal cheese *Thermobacterium helveticum* (*B. casei* ε) which produces a certain amount of propionic acid, is the predominant organism according to Orla-Jensen. The lactic acid bacteria do not act directly on the casein, but by means of proteolytic enzymes which are released by autolysis of the dead bacterial cells. These enzymes, in conjunction with the enzymes present in the rennet, bring about the progressive hydrolysis of the paracasein which constitutes the ripening. The characteristic flavours are probably produced by specific organisms.

Roquefort, Gorgonzola and Stilton are other varieties of hard rennet cheeses. The principal organism concerned in the ripening of these cheeses is the *Penicillium roqueforti*, although a certain degree of ripening, particularly in the early stages, may be due to the enzymes of the bacteria and the rennet. The piquant taste of these cheeses is due to the products of hydrolysis of the fat by the enzymes elaborated by the mould.

The soft rennet cheeses are generally made small in size, and of such shape that they have a large surface in proportion to their volume, so that the ripening agent can diffuse in more readily. Camembert cheese, which is of this type, retains considerable moisture in the curd, and a high acidity develops as the lactic acid bacteria continue to ferment the lactose. The conditions are then favourable for the growth of moulds, and *Oidium lactis* appears, soon followed by *Penicillium camemberti*. These reduce the acidity and produce proteolytic enzymes which diffuse into the cheese. The products of protein hydrolysis neutralize part of the acid. When the whole of the curd has been attacked by the proteolytic enzymes of the moulds and the rennet, and the acid is all neutralized, the ripening is completed. After this point the conditions are right for the growth of putrefactive bacteria, and the cheese may be quickly ruined.

In the ripening of all cheeses, the conditions of temperature and humidity must be very carefully adjusted in order to attain that equilibrium of the microflora which will produce the desirable result.

BACTERIOLOGICAL EXAMINATION OF CHEESE.

The sequence of changes in the bacterial flora of cheese may be followed by bacteriological examination of the cheese at appropriate intervals. Since the organisms are not always evenly distributed throughout the whole cheese, it may be necessary to examine samples from various parts. The samples are cut out, weighed, and ground up with a definite quantity of sterile water. The suspension thus obtained may be examined by the methods used in the examination of milk or butter, and the number of organisms per gramme calculated.

The bacterial flora of cheese may also be studied by direct microscopic examination. Small sections of the cheese are fixed in alcohol, and cut in paraffin. The method was devised by Troili-Petersson (1904) and has been used for various cheeses by Harrison (1906), Hucker (1921^2), Sadler (1926), and others. Hucker has applied the method to the enumeration of bacteria in cheese, but the uneven distribution of the bacteria make it necessary to count numerous fields to obtain reliable results.

DEFECTS OF CHEESE.

The bacterial flora existing in cheese at any stage depends not only on the flora of the original milk and the 'starter' culture used in producing the desirable acidity, but is intimately bound up with the processes to which the cheese is subjected in the course of manufacture. Variations from the characteristic properties amounting to defects in any cheese may be due to any fault in the manufacturing process, which adversely affects the bacterial equilibrium. Defects other than these may be due to undesirable organisms in the original milk, or to contamination at some later stage. The most important of these organisms are probably the gas-producing bacteria of the *colon-aerogenes* group, which may be the cause of 'sponginess', or 'blown' cheese, and which may produce unpleasant flavours. Putrefying bacteria produce undesirable flavours when the acidity is reduced during the ripening stage.

Discoloration may be due to various chemical reactions with metals from the utensils, but may also be produced by chromogenic organisms. Connel (1897) found that 'rusty spots' in Cheddar cheese are caused by *B. rudensis*. Blue spots in Edam cheese are due to *B. cyaneofuscus*.

Bacteriology of Special Dairy Products.

Concentrated Milk.

Milk may be evaporated to varying degrees, and after partial or complete sterilization may be kept indefinitely. As in the case of all other dairy products the best results can be obtained only when a high grade clean milk is used.

Condensed milk. The milk is evaporated *in vacuo* to about one-third of its bulk. Cane sugar is added before evaporation so that the final product contains about 30 to 40 per cent. of sugar. It is put in cans and sealed. Condensed milk is not sterile, but bacterial development is held in check by the high concentration of sugar. Some of the bacteria in the original milk, including a few micrococci, spore-forming bacteria, yeasts and moulds may survive. The yeasts may produce gas, causing the tins to swell. Moulds may grow to a limited extent and produce proteolytic enzymes which attack the casein.

Unsweetened evaporated milk. The milk is evaporated *in vacuo* to about one-third of its volume, put in cans, sealed and sterilized by steam under pressure. If the sterilization is complete, the milk will keep indefinitely. A few sporing forms and thermobacteria may survive and produce peptonization, or partial coagulation of the milk.

Dried milk. Milk may be preserved by evaporation to complete dryness. This is more easily attained with skim milk than with whole milk. Pasteurized milk, evaporated to about half its original volume, is sprayed into a chamber where it meets a current of air at 120° C. The drying is completed as it falls to the bottom, and the dry powder is collected and hermetically sealed in tins. Milk powder prepared by this method is more readily soluble in water than milk powder prepared by other methods in which it is subjected to a higher temperature. The milk powder is not sterile, but no bacterial growth takes place in the dry powder.

Details of these and other methods of preserving milk, and the bacteriology of the products, may be found in the authoritative work of Hunziker (1920).

Ice Cream.

Ice cream is an important dairy product consisting of cream, condensed milk, sugar, gelatin and flavouring substances in varying proportions. Its bacterial content depends largely on that of the cream and condensed milk, and is very variable. The putrefactive types will develop relatively more rapidly than the lactic acid types at the low temperature of the ice cream, and if it is kept long before use it may contain objectionable products of bacterial metabolism. Pathogenic organisms are sometimes found in ice cream made from unpasteurized cream.

Fermented Milk Drinks.

Fermented milk beverages have long been used in various parts of the world because of their reputed dietetic or therapeutic value. Some of these are of considerable interest from the bacteriological point of view.

Yoghurt, used by the Bulgarian peasants, is a sour milk in which certain aromatic substances are produced by *Saccharomycetes.* The lactic-acid-forming streptococci, and the more vigorous lactic-acid-forming bacteria produce the desired degree of acidity in the milk. *B. bulgaricus* (*Thermobacterium bulgaricum*, Orla-Jensen, 1919) is the principal lactic-acid-forming organism in Yoghurt.

Leben is an Egyptian product, prepared from the milk of cows, buffaloes or goats. It contains lactic acid bacteria, and yeast or torulae which ferment the lactose to alcohol and lactic acid. The alcoholic fermentation is rather more important than the acid in Leben.

Kaeldermaelk is made in Norway from boiled milk inoculated with a special variety of ropy milk. The fermentation goes on at a low temperature, producing a considerable amount of lactic acid, and a small amount of alcohol. The product is stable, and may be kept for several months.

Kumys is made in Russia from mare's milk, which has a high sugar content. It is fermented by a yeast in conjunction with lactic acid bacteria, and the final product may contain 3 per cent. of alcohol and 1 per cent. of lactic acid.

Kefir is made by the inhabitants of the Caucasus Mountains. It is prepared by inoculating milk with Kefir grains. These curious grains, resembling small cauliflowers, are produced by the symbiotic growth of the organisms concerned in the fermentation. On section, they are seen to consist of a network of bacteria in which yeast cells are enclosed. When inoculated into milk, the grains increase in size during the fermentation, and fresh grains are produced. Kefir is an effervescent fluid containing lactic acid and alcohol. The lactic acid bacteria in Kefir are chiefly *Betabacterium caucasicum* (Orla-Jensen, 1919).

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CHAPTER IV. THE BACTERIOLOGY OF FOODS (OTHER THAN MILK AND MILK-PRODUCTS).

By William G. Savage.

Meat, Meat-products and Fish.

It is generally accepted, largely on the strength of German work carried out between 1909 and 1912, that the musculature of healthy, freshly slaughtered animals is sterile and that the positive results which are occasionally obtained are due either to contamination during the manipulation of slaughtering or to faulty technique. The recent work of Reith (1926) is not in accord with this view. Of 24 hams examined within 1 hour of slaughter, 77 per cent. contained aerobic and 57 per cent. anaerobic bacteria. Many of the organisms did not develop until after 7 to 14 days. Muscle tissue, removed under an anæsthetic, from living animals showed aerobic growths in all 36 samples from 6 hogs, and from 61 and 89 per cent. respectively of the rabbits and guinea-pigs, while numerous positive results were recorded with anaerobic media. Further confirmation is necessary before this can be accepted.

The internal solid organs of freshly killed healthy animals frequently have been shown to contain a small number of living bacteria, both aerobic and anaerobic, sometimes so scanty as only to be demonstrable when enrichment methods are used. It is probable that many are post-mortem invasions or have entered just before death. The chief value of the observation is that this possibility must be considered before it is assumed that the isolation of an organism from such organs has any pathological significance.

Organisms are invariably present on the surface of market meat even when fresh, but, as Gaertner demonstrated in 1908, they do not penetrate appreciably for several days. The numbers on and just beneath the surface increase with great rapidity, the three controlling factors being the amount of handling of the meat, the temperature at which the meat is maintained and the time interval. The bacilli penetrate between the muscle fibres deep into the meat. Numerous studies have also been made of meat sold in fragments and small pieces as purchased in shops and markets, and aerobic bacteria have been found to vary from 270,000 to 88,000,000 per gramme (Weinzirl and Newton, 1914). Attempts have been made to use an estimate of the number of aerobic bacilli present as a measure of the hygienic conditions of the meat, including the time since slaughter and the conditions under which kept, but it is difficult to devise a satisfactory enumeration method, and the factors influencing the multiplication of bacteria are so many that it is impossible to set up any useful standards.

The bacterial content of made-up meat products such as brawn or sausages varies greatly. Foods such as meat pies and brawn should be comparatively sterile, owing to their being cooked after preparation. The presence of a large number of organisms indicates either inadequate heating or unsatisfactory methods of storage and treatment subsequent to heating. Sausages usually contain large numbers of bacteria, including *B. coli*, the latter being found by Savage (1908–9) to vary from 100 to 100,000 per gramme.

Fresh fish is usually sterile as regards the interior of the flesh, but numerous organisms are present on the surface, even of freshly caught fish. These bacteria rapidly increase under ordinary conditions and penetrate into the muscles. Fellers (1926) in a study of raw salmon spoilage found numerous organisms in the mouth, gills and slime of live salmon. He found that bacteria penetrate the flesh under average conditions in from 24 to 60 hours, depending upon such factors as the size and species of the salmon, temperature and methods of handling. The number of bacteria in the flesh increase rapidly with each 24 hours. Hunter (1920) had previously obtained similar results, and after 96 hours at temperatures between 50° F. and 70° F. the count of bacteria in the muscle tissues reached as high as 155,000,000 per gramme.

These facts and a consideration of the kinds of bacteria present are mainly of interest in connection with the question of the spoilage of meat and fish. Broadly considered, spoilage may be putrefactive or non-putrefactive. Non-putrefactive spoilage includes such conditions as mouldiness, due to the growth of many different kinds of moulds, souring caused by saprophytic acid-producing bacteria, and special abnormal changes, of which local colour changes are the most striking. Good examples of the last are red discolorations on bread and other foods from *B. prodigiosus*, and Pink Eye on dried salted fish due to two organisms—a red sarcina and an unnamed polymorphous organism (Cloake, 1923). These abnormal conditions are of considerable trade importance. Spoilage due to putrefactive changes is of immense commercial importance and merits more detailed consideration.

Bacteriology of putrefaction. By putrefaction is understood the decomposition of organic matter, chiefly protein in nature, by the action of bacteria, by which it is split up into a number of chemical substances, many of which are foul smelling. The evidence favours the view that the actual production of these foul-smelling bodies is the work of anaerobes only, while the action of aerobes is mainly digestive and preparatory. If, therefore, the expression putrefaction is taken as including the whole range of the changes from the preliminary hydrolysis of the protein molecule to the production of these offensive bodies, we have to accept both aerobes and anaerobes as taking part.

The aerobic organisms concerned in the process are very numerous. Acid-producing micrococci can initiate important changes in salmon (Fellers, 1926). The sporing aerobes which possess proteolytic properties such as *B. subtilis*, *B. mesentericus*, *B. vulgatus* and many others play a more active part (Savage, Hunwicke and Calder, 1922). Organisms of the *B. coli*, *B. cloacæ*, *B. fluorescens liquefaciens* groups are usually abundant and initiate decomposition changes. The bacteria of the *B. proteus* group are the aerobes which seem to be able to carry protein degradation further than any other aerobes, and their decomposition products include ammonia, sulphuretted hydrogen, indole and indole acetic acids, all of which are associated with putrefaction. Most of these organisms (and practically invariably *B. proteus* and *B. coli*) can be readily isolated from decomposing meat or fish.

It is, however, to certain groups of anaerobes that the most profound decomposition changes are due. These are sporing proteolytic anaerobes of many kinds, of which *B. sporogenes* and *B. putrificus* are well-known examples. The protein degradation products which result include bodies such as indole, skatole, skatole-acetic acid and phenol, and simpler substances like ammonia, methane, carbon dioxide, sulphuretted hydrogen and hydrogen. In addition certain definite poisonous bases such as β imidazolethylamine and tyramine are produced by the action of putrefactive bacteria upon the amino-acids histidine and tyrosine. The ptomaines such as putrescine, cadaverine and neurine are also bodies which may be formed in the later stages of putrefaction. The ptomaines are chiefly of historical interest, since the evidence that they are associated with the causation of food poisoning is now discredited.

A reliable bacteriological test or technique for the detection of incipient putrefaction would be of considerable administrative utility. There is no relationship (Hoffstadt, 1924, and others) between the total aerobic bacterial count, a count of glucose fermenters, or one of proteolytic aerobes and either the fitness of the meat or the degree of early spoilage. Hoffstadt has suggested that the presence of proteolytic anaerobes might be used to indicate how the keeping qualities of meat may be predicted, but no reliable test has yet been evolved.

Shellfish.

Shellfish taken from specifically contaminated layings have conveyed to man typhoid fever, paratyphoid fever, acute food poisoning and other intestinal infections. The shellfish incriminated are oysters, mussels and cockles, the first-named being the most important.

Bacteriological investigations are employed for three purposes:

(*a*) To pass judgment on particular batches of shellfish, as regards potential danger to health.

(*b*) To enable an opinion to be given as to the safety of any given shellfish layings.

(*c*) To study the conditions necessary to free dangerously contaminated shellfish from their bacterial pollution and make them safe for human consumption.

Examination of Shellfish.

Oysters. As the result of numerous examinations it has been possible to set up working standards as to the number of organisms indicating excretal or sewage pollution which are permissible in oysters. The indicator organisms used are *B. coli*, streptococci and *B. welchii*. Houston's (1904[1]) standards per oyster were :

B. coli	100 stringent, 1,000 lenient.
B. welchii	10 ,, , 100 ,,

Another standard is based upon Klein's method of examination, 0·2 c.cm. of shell liquor from each oyster being examined for *B. coli*. This amount is approximately equivalent to 1/50 of an oyster, so that a positive result means at least 50 *B. coli* present per oyster, and, according to Klein's experimental results, usually about 200 *B. coli* per oyster. Ten oysters are examined in this way (see below) and the results are returned as a *percentage* clean, according as they pass this test, i.e. all 10 oysters free from *B. coli* (in 1/50 oyster) = 100 per cent. clean, 5 showing *B. coli* = 50 per cent. clean, 10 containing *B. coli* = 0 per cent. clean. This is the standard adopted by the Fishmongers' Company and approved for adoption by a committee of bacteriologists (Andrewes, Hewlett and Eyre, 1924). It approximates to the stringent standard of Houston. This short method of examination is adequate for routine work but a very exact technique is necessary (see Eyre, 1924). More detailed and comprehensive methods for research or special purposes are described by Houston (1904[2]) and by Eyre (1924). Streptococci and *B. welchii* counts are mainly used to corroborate the more reliable *B. coli* enumerations.

Mussels. Examinations by Buchan (1908), Johnstone (1913), and others, show that these shellfish contain numerous *B. coli*, and usually more numerous than in oysters, but available data are inadequate to justify limiting standards being set up. A number of outbreaks of food poisoning, characterized by a peculiar symptomatology and often a high case mortality, have been recorded on the Continent and in the British Isles following the consumption of contaminated mussels. Brieger (1885) isolated a poisonous base, *mytilotoxine*, from contaminated mussels in the Wilhelmshaven outbreak and this substance is usually thought to be the toxic agent. Whether it is so is, however, doubtful, and it cannot be accepted as such without further evidence. Very few outbreaks have been recorded in recent years, so no opportunities have been available to re-investigate their pathology.

Cockles. These are often heavily contaminated with *B. coli*, but systematic analyses are even fewer than for mussels.

Shellfish Layings.

It is sometimes necessary to study the bacteriological conditions of oyster and other shellfish layings to ascertain if they are satisfactory and permissible for shellfish culture, in addition to making a careful topographical investigation of sources of pollution. Three kinds of material are available for examination. One is the shellfish. In this connection it may be mentioned that American workers (Gorham, 1912, Round, &c.) attach considerable importance to temperature conditions as influencing the number of bacteria present—a point worthy of consideration. Round (1916) found the proportion of *B. coli* on the body of the oyster, compared to the number in shell liquor, to be higher the warmer the water. The sea water which flows over the oysters is often sampled, and since deep-sea water is free from these excretal indicator organisms, while sewage-contaminated sea water contains them abundantly, results of considerable value are theoretically obtainable. In actual practice these examinations are not very helpful owing to the great fluctuations and movements of the sea water at tidal outlets and the marked effect of depth on contamination. The third material is the mud in these tidal estuaries, and Savage (1905) found it a reliable measure of the degree of local contamination. The polluted muds showed 10,000 to 100,000 *B. coli* per gramme of mud where most polluted and less than 10 per gramme in the unpolluted creeks. If the muds show a relatively high purity they indicate a safe fattening area for oysters.

The Self-purification of Oysters.

The bacterial contamination of oysters is markedly reduced by their transference to pure running water. Klein, in 1905, infected oysters with very large numbers of typhoid bacilli (using a laboratory culture), and after eleven days' sojourn in pure water these bacilli were absent from one-eighth of an oyster. When the pollution is estimated in terms of *B. coli* the reduction is also rapid, but complete freedom is less readily attained. Carnwath (1909), from a limited number of experiments, found a 99 per cent. reduction of *B. coli* in infected mussels when placed in pure water.

The problem, one of considerable commercial importance, as to whether oysters removed from polluted sources can be rendered safe for human food by a sojourn in pure-water tanks, or by other means, must still be considered *sub judice*. For instance, Wells (1926) states that polluted oysters (in Vancouver) transferred to pure water eliminate almost all the contained organisms within a few hours at feeding temperatures and within a few days in the winter. Tarbett (1926), Wells and other American workers advocate the self-purification of oysters by allowing them to cleanse themselves in water treated with chlorine. Krumwiede, Park, Cooper, Grund, Tyler and Rosenstein (1926[1 & 2]), on the other hand, have obtained less promising results from a long series of observations upon oysters artificially contaminated with typhoid bacilli, using very thorough and modern methods for the detection of these bacilli. Under refrigerator temperatures the typhoid bacilli persisted for as long as the oysters lived—a period of 41 days. They were isolated up to 21 days from artificially contaminated oysters placed in sea water which was frequently changed. They still persisted for long periods when chlorinated water was used. These authors conclude : ' The chlorination treatment of contaminated oysters will result in a marked diminution in the number of *B. typhosus,* but even six successive treatments may not rid the oyster of the contaminating pathogens. The results indicate that safe oysters can only be produced by growing them in waters uncontaminated by human fæces '.

The Bacteriology of Eggs.

The bacterial content of perfectly fresh eggs. The literature is extensive, but the published results show considerable discrepancies, probably in part due to faulty technique and varying methods, in part because the eggs examined may not always have been fresh. The results suggest that a definite proportion, which is about 8 to 10 per cent., of fresh eggs is not sterile and that the bacteria are mostly present in the yolk. Maurer (1911) examined 600 fresh eggs and found that 18·1 per cent. contained bacteria. Of the infected eggs 82 per cent. were infected in the yolk, 25·9 per cent. in the white and 7·9 per cent. in both yolk and white. Bushnell and Maurer (1914) examined 2,759 eggs and found 23·7 per cent. infected with bacteria. Rettger (1914) obtained lower figures with improved technique and his percentages positive decreased from 7·7 and 8·9 in 1911 and 1912 to 5·6 in 1913. Hadley and Caldwell (1916) examined 2,520 fresh eggs, of which 8·7 per cent. showed yolk infection. Only 111 whites were examined and none showed any bacteria. The percentage of infection with the eggs of individual hens varied between 2·8 and 15·0, the average being 9·0. The bacterial varieties have not been worked out in detail in most of the investigations. Many are cocci of various kinds and coccoid forms. The proteolytic and fermentative groups, such as *B. coli, B. proteus,* &c., do not seem to be present in fresh eggs, and the decomposition of eggs does not appear to be related to the bacteria in the yolk of fresh eggs. The higher bacterial infectivity of the yolk suggests infection before the egg reaches the oviduct and the white is deposited, but the evidence is not conclusive as to whether bacterial infection occurs in the ovary or in the oviduct.

According to most investigators (Rettger and Sperry, 1912, and others) egg white has antiseptic properties, but other workers deny this (Poppe, 1910; Bainbridge, 1911; Maurer, 1911, &c.). The view of Poppe (1910) that the eggs of unfertilized fowls are sterile and the fertilized may contain bacteria is often quoted, but rests on very slender evidence. Poppe only examined eight fresh eggs. Hadley and Caldwell (1916) found the percentage of infection in the two classes to be the same. On the other hand, ovarian infection of eggs has definitely been shown to occur with

B. pullorum, the bacterial cause of white diarrhœa of chicks (Rettger, 1914). In this disease the ovary is the important seat of infection. There is no evidence that this bacillus is pathogenic to man.

The permeability of eggs to bacteria and moulds. The shell of the egg is porous not only to gases but to sufficiently small solids. According to Rettger (1914) the shell of a fresh egg is coated with a thin layer of a gelatinous substance which under normal conditions protects the egg from bacterial invasion. Damp and soiled eggs soon become germ-permeable. Moisture facilitates mould invasion. The shells of eggs are never sterile, the numbers of bacteria present varying with their cleanness. Pennington (1914) found the bacteria to range from 400 to 1,600,000 per gm. and *B. coli* from 10 to 10,000. *B. coli* can usually be isolated. That bacteria can penetrate sound eggs under favourable circumstances has been shown by numerous workers. The usual procedure is to take washed sound eggs and immerse them in solutions containing the bacilli under investigation. In this way the penetration of *B. typhosus* (Piorkowski, 1895), cholera vibrios (Wilm, 1895), *B. paratyphosus B.* (Poppe, 1910), *B. enteritidis, B. coli, B. paratyphosus, B.*, and *B. typhosus* (Lange, 1907), *B. proteus* (Kossowicz, 1913), has been demonstrated. With the bacilli smeared on the shell other workers have not been successful (Maurer, &c.). Probably, under natural conditions, penetration of bacteria does not readily occur.

The bacterial decomposition of eggs. No recent bacteriological investigations appear to have been made, and the descriptions of special organisms in the older bacteriological reports are of no value. Anaerobes are not usually mentioned. Unsound eggs may be either mouldy or decomposed. Mouldy eggs are not putrefactive. Almost any moulds can penetrate eggs given sufficient moisture on the surface and a suitable range of temperature. The putrefactive bacteria are probably the same as those met with in highly nitrogenous foods (see p. 75), but possibly various sulphur-decomposing types predominate.

Preserved eggs. Eggs may be preserved by the use of chemicals, be dried or frozen. Dried eggs usually show the presence of large numbers of bacteria. Ross (1914), for example, examined 248 samples and 173 (70 per cent.) contained 100,000 to 500,000 organisms per gm. Only 6 were below 10,000, while in 18 there were 500,000 to 1,000,000 per gm. Pennington (1916) found the average count for 48 samples to be 3,600,000, the counts ranging from 65,000 to 20,000,000 per gm. The number of *B. coli* per gm. varied from 0 to 1,000,000. The dried product contained about three times as many bacteria as the liquid egg. Frozen eggs show equally heavy bacterial contamination. Stiles and Bates (1912) prepared frozen eggs in the laboratory, using low-grade eggs, and the product showed bacterial counts ranging from 1 to 10,000,000 bacteria per gm. With second-grade eggs the bacterial counts were usually less than 1,000,000 per gm.

The bacteriological examination of eggs. The exact steps will vary with the special purpose in view, but some general points may be mentioned. Very great care is essential to prevent contamination from bacteria on the outside of the shell. This can be done by washing in water, treating with antiseptic (0·1 per cent. mercuric chloride), washing in alcohol and thorough flaming of the part to be opened. Part of the shell is removed and the white and yolk removed by separate pipettes. To test sterility, the whole of the white and the yolk as far as possible should be removed and mixed with broth in suitable flasks or wide test-tubes. Different culture media can be inoculated from these broth-culture tubes. Several incubation temperatures should be used (21°, 37° and possibly 55° C.).

Vegetables and Fruits.

The special bacterial diseases of vegetables are outside the scope of this section. Vegetables are usually soil-contaminated, and organisms are readily isolated from their surfaces. *B. coli* and other excretal types are frequently abundant when there has been much manuring of the market garden. Kozyn (1908) has frequently isolated *B. tetani* from vegetables. When not affected with one of the special bacterial diseases the interiors of vegetables are sterile.

Bacteria, moulds and yeasts are common on the surface of fruit, and are present even on fruit taken direct from the tree (Ehrlich, 1901). When the fruit is obtained from a market the numbers are always much greater and comparative results show higher figures from dirty, dusty stalls than from clean. Numerous investigations (Mills, Clifford, Bartlett and Kessel, 1925, and others) have shown that sound fruits are sterile under the skin, while if bacteria are inserted into cut surfaces they tend to die out. When fruit is stored the extent of deterioration from micro-organisms largely depends upon the conditions of storage, particularly temperature and moisture. If condensation water collects on the surface the mould spores which are invariably present will germinate and mycelial penetration results, with mouldy deterioration.

The viability on fruit and vegetables of intestinal pathogenic bacteria, such as *B. typhosus*, *V. choleræ* and *B. dysenteriæ*, has been tested by a number of workers, for example Bindseil (1917), Mackie and Trasler (1922), Vasquez-Coht (1924), and found to be from a few days to as long as 50 days or more. Schnirer (1891) claims to have cultivated tubercle bacilli from grapes bought in a market opposite a tuberculosis clinic. Watercress taken from layings contaminated with human excreta may convey enteric fever. As Houston (1905) has shown, watercress itself may be contaminated with very large numbers of *B. coli* and other excretal indicators when grown in polluted waters, while the waters in which it is grown may contain as many as 1,000 to 10,000 *B. coli* per c.cm.

Fresh fruits or vegetables very rarely act as vehicles for the spread of food poisoning. Potatoes have been incriminated in a number of outbreaks, many of which are ascribed to solanin poisoning, but it is likely that bacterial infection is a commoner cause.[1] Canned fruits and vegetables have in a few cases been associated with Salmonella food poisoning, and in many instances with outbreaks of botulism (see Food Poisoning, p. 408).

Bread.

Bacteriologically two problems are of interest, i.e. bread as a vehicle for the conveyance of bacterial infections and anomalies of bread due to the activities of undesirable bacteria. Since bread is subjected to a good deal of handling it is not surprising that the numerous examinations which have been made usually disclose considerable bacterial contamination. Washings from the surface of unwrapped loaves have yielded figures varying from 2,000 to 3,000,000 or so bacteria per loaf (Howell, 1912, &c.). The numbers are comparatively low since appreciable multiplication on the surface does not occur. Outbreaks of infectious disease traced to bread as the vehicle are rare, but Furst (1914) considered that an outbreak of enteric fever in 1913 in a children's institution was spread in this way, the primary source of infection being from enteric fever cases amongst the family of the baker. Lumsden and Anderson (1911) also ascribed several cases of enteric fever in a hospital for the insane at Washington to an attendant who cut up the bread and also was in charge of a typhoid patient in his home.

Bread is subject to several microbial infections which damage it as a commercial product. Among breads so damaged are mouldy bread, due to the growth of different kinds of moulds, red bread, coloured with *B. prodigiosus* and chiefly of historical interest, and 'ropy' bread. The expression 'ropy' bread is not a term with any exact precision, but indicates a sticky deterioration of the crumb, often a brown discoloration and an unpleasant odour, and a change in chemical reaction, which may be markedly acid or markedly alkaline. Many bacteria have been isolated from 'ropy' bread and described by different investigators under special names. Probably more than one type of organism may be concerned, but Lloyd, Clark and McCrea (1921) in a study in England found all the six types they isolated to belong to the *B. mesentericus* group. They found organisms of this group present in the skins of all grains, all flours and all bread. Rope only develops when favourable conditions are present and these are—a high degree of infection, moisture, a suitable temperature, a suitable chemical reaction, and to some extent flour of a favourable composition.

The Bacteriology of Preserved Foods.

The preservation of foods is a very ancient practice, but the methods have been purely empirical, and only comparatively recently have attempts been made to study the subject scientifically. Accurate bacteriological data are very incomplete. The different methods used may be grouped as preservation by (*a*) drying, (*b*) the use of cold temperatures, (*c*) the use of chemicals, (*d*) the action of heat and storage in air-tight receptacles. Some methods (for example, smoking foods) utilize more than one of these procedures.

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[1] For a full discussion, see Savage, '*Food Poisoning and Food Inspection*', 1920, pp. 141–8.

Drying.

The food is so altered, either by absence of sufficient moisture or by concentration of the solute, that it will no longer serve as a nutrient medium for the growth of bacteria. The food is not rendered bacteria-free, but organisms present are unable to multiply and so to decompose the product. Usually the actual numbers present are inconsiderable. For example, with vegetables and fruits, Prescott (1920) reported numbers per gm. from about 100 to 300,000. Fungi can thrive in media with much less water than bacteria, and with cane sugar it has been shown that deterioration from the action of moulds may result with as little as 1 per cent. of water present. Satisfactory storage of dried products is almost entirely a question of preventing increase of moisture.

Cold.

Refrigeration problems are not entirely a question of temperature but are concerned with the types of bacteria present and the nature of the material cooled. For example, with a highly nutritive food such as milk, held at 0° C., for the first few days there is no bacterial increase, while even a decline in numbers may occur. A very gradual increase subsequently takes place which may reach a thousandfold multiplication after several weeks. A selective bacterial action also occurs. The same slow growth of bacteria on the surface of meat stored just above freezing (32 to 36° F.) was shown by Hoagland, McBryde and Powick (1917) with but very trifling bacterial penetration.

Cold is useless as a sterilizing agent: MacFadyen and Rowland (1900) showed that pathogenic bacteria of various kinds could resist -182 to $-190°$ C. for 7 days. These results were purely qualitative and marked diminution in numbers may have resulted. Indeed a number of other workers have shown that different organisms, including pathogenic bacteria, gradually die out at temperatures below 0° C. Factors other than cold play a part, such as intermittent freezing and thawing, the presence of colloidal or solid bodies which offer physical protection, and the mechanical effect of crystallization. A further factor inhibiting growth is that below the freezing point of the material no water is available to enable multiplication of bacteria. With temperatures a little above the freezing point of the substance, moisture is available and slow bacterial multiplication occurs. With such a food as meat putrefactive changes can occur from the slow development of proteolytic bacteria.

Chemical Action.

Chemicals are extensively used either in connection with practices of considerable antiquity, such as using salt, salt and nitre, or sugar, or the more modern employment of chemical substances with definitely germicidal properties, such as boric acid and salicylic acid compounds, benzoic acid and formaldehyde. In the strength with which they are found in foods their action is inhibitory rather than germicidal. No definite selective action as regards their action on different groups of bacteria has been proved.

The effects of salt solutions on bacteria merit more consideration. In ordinary salt-pickling solutions bacteria are numerous. Many workers have shown that even in solutions containing up to 25 per cent. of sodium chloride bacteria can be isolated and will even slowly increase in numbers. Such solutions have a selective action on certain types of bacteria as Pettersson (1900) showed many years ago. For ordinary bacteria a 10 per cent. sodium chloride solution exerts a considerable inhibitory effect, but certain strains will grow in 20 per cent. or even more concentrated solutions. These strains have been termed halophilic organisms, but very little is known about them or whether they are distinct types. Some yeasts will flourish in 18 to 20 per cent. solutions. The growth of most pathogenic bacteria is inhibited, but behaviour of Salmonella food-poisoning bacilli is of interest. Savage (1910) found a rapid elimination in 15 per cent. sodium chloride solutions, but in one experiment, after initial diminution, a strain survived for 38 days. Weichel (1910) obtained somewhat similar results and also recorded that with meat already infected with Salmonella bacilli, solutions of salt up to 19 per cent. failed to kill the bacilli within a reasonable time (survival up to 75 days). Karaffa-Korbutt (1912) reported an inhibitory effect with 6 to 7 per cent. solutions, but Serkowski and Tomezak (1911) found solutions below 10 per cent. had only a feeble inhibitory action and that a strength of 15 to 20 per cent. was necessary. The inhibitory action of salt is usually ascribed to the partial dehydration of the proteins and other substances, but Rockwell and Ebertz (1924) consider that a part is played by its ability to exclude oxygen and its interference with the action of proteolytic enzymes.

Canning.

The foods, after various initial treatment, are placed in air-tight receptacles—tins or bottles. The containers are closed and then subjected to heat with the object of destroying all living bacteria in the food. With sweetened condensed milk sterility is not aimed at, the development of bacteria being prevented by the high proportion of sugar. A great many technical points have to be attended to if satisfactory results are to be obtained. An enormous number of different foods have been preserved by these methods.

Absolute sterility is not necessary for the maintenance of soundness. The following table (Savage, 1923) gives the results of the examination of perfectly sound samples of various canned foods purchased in England in the open market:

Product.	No. examined.	Sterile.	Not sterile.	Percentage not sterile.	Percentage not sterile.	
Meat	22	8	14	63·6		
Salmon	16	8	8	50·0	Fish 48·6	Marine Products 61·1
Sardines	11	7	4	36·4		
Herrings	8	3	5	62·5		
Crab	10	0	10	100·0	Crustacea 84·2	
Lobster	6	1	5	83·3		
Crayfish	3	2	1	33·3		
Fruit	58	45	13	22·4		
Unsweetened condensed milk . .	44	36	8	18·2		
Sweetened condensed milk . .	15	0	15	100·0		
Total (excluding sweetened milk) . .	178	110	68	38·2		

The kinds of organisms isolated from the above samples, omitting those from sweetened milk, are shown in the following table (Savage, 1923):

Product.	Yeasts.	Obligate anaerobes.	Sporing aerobes.	Thermophils.	Micrococci.	Non-sporing bacilli.
Meat	0	0	7	6	5	1
Salmon	0	0	3	3	4	1
Sardines	0	0	4	1	1	0
Herrings . .	0	0	3	3	0	0
Crab	0	2	2	9	3	0
Lobster and Crayfish . .	0	0	4	2	0	0
Fruit	1	7	10	0	0	0
Unsweetened milk	1	0	5	1	4	0
Total . .	2	9	38	25	17	2

Some tins of canned foods become unsound owing to bacterial development. This may result from the introduction of bacteria from without through leaks, such as may occur with faulty seams or rust erosion, or from the development of bacteria already present, but dormant, which become active from various causes. In either case the bacteria proliferate and decompose the food. The following table (Savage, 1923) gives particulars of the bacterial content of 257 unsound tins examined by Savage, Hunwicke and Calder:

	Food product.									
	Meat.	Salmon.	Sardines.	Herrings.	Lobster.	Crab.	Crayfish.	Fruit.	Sweetened Milk.	Unsweetened Milk.
No. of tins examined	32	27	16	5	9	13	8	67	33	47
Types of organisms isolated:										
Anaerobes	9	2	3	0	2	3	1	3	1	4
Sporing aerobes (excluding thermophils)	11	9	4	2	2	4	3	9	33	7
Thermophils	4	1	4	0	1	1	0	3	10 (out of 16).	0
Non-sporing bacilli of decomposing type	5	11	4	0	3	3	1	2	5	6
Non-sporing bacilli of non-decomposing type	6	3	4	0	0	5	0	0	—[1]	2
Cocco-bacillus type	0	0	0	0	0	0	0	16	—[1]	5
Micrococci	13	10	3	4	3	7	1	14	33	21
Yeasts	0	0	0	0	0	0	0	12	30	2
Sterile	2	3	5	1	1	0	3	24	0	9

[1] Not looked for in every sample.

Organisms incapable of producing either gas or initiating protein decomposition do not usually contribute to unsoundness. The organisms responsible for producing decomposition vary with the type of food. The following results were obtained from English work carried out in 1920–23:

Meat and marine products. Savage, Hunwicke and Calder (1922) found decomposition due to proteolytic anaerobes, proteolytic sporing aerobes and non-sporing aerobes which are proteolytic, fermentative, or both. Such organisms include *B. proteus, B. cloacæ and B. coli.* No decomposition was associated with yeasts, aerobic bacilli devoid of proteolytic or carbohydrate-fermenting properties, micrococci or thermophilic bacteria.

Sweetened condensed milk. Savage and Hunwicke (1923[1]) found gaseous decomposition (blown tins) to be due to yeasts in over 90 per cent. of cases. Proteolytic sporing aerobes may occasionally play a part. No evidence was obtained that fermenting non-sporing bacilli of *B. coli* type, anaerobes, micrococci or thermophilic bacilli act as decomposing organisms.

Unsweetened condensed milk. Savage and Hunwicke (1923[1]) found Gram-positive micrococci of various kinds to be a common cause of decomposition, and some of their strains could produce gas. Decomposing organisms less frequently met with were anaerobes, yeasts, gas-producing non-sporing bacilli (*B. coli*, *B. cloacæ*, *B. proteus*, &c.) and *B. pleofructi*.

Fruit. The decomposing organisms found by Savage and Hunwicke (1923[2]) were *B. pleofructi*, micrococci, yeasts and non-sporing bacilli of fermenting type. Sporing aerobes, anaerobes and thermophilic bacilli were not found as a cause of decomposition. The name *B. pleofructi* was given to an organism, or rather a group of nearly identical strains, with peculiar features. It is highly pleomorphic, growing in broth or on agar as a very short bacillus only slightly longer than broad and easily mistaken for a coccus, found in pairs or quite short chains, while in fruit juice it forms long, slender bacilli. It is Gram-positive, non-motile, capsulated, and without spores. It grows well under both aerobic and anaerobic conditions and at both 21 and 37° C. It does not liquefy either gelatin or blood serum. It is non-pathogenic to mice. While none of the strains produce gas in ordinary media they differ among themselves as regards acid production from glucose, saccharose and lactose. Some strains in milk produced acid and clot, others did not. On the other hand, when introduced into sound tins of fruit, definite amounts of gas were produced.

The above results differ in detail, but not in their broad outlines, from those obtained by a number of workers in U.S.A., for example Weinzirl (1919), Cheyney (1919) and Fellers (1926), the last-named working with canned salmon. Cameron and Esty (1926) have shown that thermophilic bacteria are a source of changes in corn and other vegetables, causing what they call ' flat sour ' spoilage.

Pathogenic bacteria are rare in canned foods, and the only ones which have been isolated are those associated with food poisoning, i.e. Salmonella Group strains and *B. botulinus*. Their presence and significance is discussed in the section on food poisoning. In the bacteriological examination of canned foods very great care must be taken to avoid outside contamination when the tin is opened. No special culture media are required, but the range of media used must be sufficient to encourage the growth of the ordinary aerobic bacilli, sporing and non-sporing, anaerobes, micrococci, yeasts and thermophilic bacteria. Three incubation temperatures should be employed, i.e. 21° C., 37° C. and 55° C. Direct (or after centrifugalization of liquid contents) microscopic examination of stained films is a useful additional procedure.

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CHAPTER V. SOIL BACTERIA.

By H. G. Thornton
(Rothamsted Experimental Station, Harpenden).

The Soil as a Medium for Bacterial Growth.

The physical and chemical conditions in the soil render it a much better medium for the life of micro-organisms than might at first sight be expected. It is built up essentially of particles held together by mineral and organic colloidal matter and forming a framework surrounding extensive pore spaces. These spaces, which may take up about 40 per cent. of the soil volume, contain an atmosphere not very different from the air above, and are lined with a film of water, spread over the particles. Evidence by direct staining methods suggests that the micro-organisms live both in this water film and also in the colloidal matter. They are, therefore, well supplied with oxygen from the atmosphere in the pore-spaces, save under exceptional conditions of water-logging, when these become filled. The mean temperature of the soil is slightly higher than that of the air and fluctuates less. The reaction in normal soils approaches neutrality and, owing to the strong buffer action of their constituents, is not easily altered. The soil water contains salts in solution. In a normal agricultural soil these appear to consist mainly of calcium bicarbonate and nitrate, salts of sodium, magnesium and potassium and only a little phosphate. The proportion of these salts varies considerably, nitrates being the most variable constituent. The small amount of phosphate in solution and its importance as a constituent of living matter make it a limiting factor to the growth of micro-organisms in some soils. The factor mainly controlling the growth of the soil population, however, is the supply of energy, which is almost wholly derived from plant residues in the soil. A normal soil contains from 2 to 5 per cent. by weight of organic matter, but a considerable proportion of this is in a form not readily available to micro-organisms.

Qualitative Study of the Soil Bacteria.

The study of the bacteria in soil may be said to date from the invention by Koch of the gelatin plate method of isolating pure cultures from a mixed population of micro-organisms. This rendered possible the separation and study of individual types. But the task of isolating and studying the enormous numbers of different organisms occurring in soil has so far proved too vast and their great diversity is beyond the scope of the various suggested schemes of classification. Moreover, it must be admitted that the recent classifications are in no way natural, since some groups of the soil bacteria have been based on morphological characters and others upon peculiarities of their metabolism. Until such inconsistencies are removed a permanent classification can scarcely result.

In number of species and diversity the soil bacteria present a difficulty to systematic survey scarcely paralleled in other branches of bacteriology. Amongst pathogenic organisms, for example, the specialized environment has greatly limited the number of forms, while their specific reactions to their hosts afford valuable means of identification. In the biological study of fermentation and other industries and in the bacteriology of milk products, the biological changes of interest can usually be attributed to a limited number of types. In the soil, on the other hand, though some of the chemical processes appear to be the work of a few specialized organisms, other changes, such as the formation of ammonia, can be carried out by an immense number of types, a systematic survey of which has not yet been made. Moreover, there is evidence that the soil, liable as it is to continual contamination from above, contains a large number of organisms, present in a resting, inactive condition. In culture media these organisms grow at least as readily as those really active in the soil.

It is a first necessity to separate the population active in the soil from the species present only in a resting condition. Attempts have been made to do this by two methods. First, the changes in the bacterial flora with changing soil conditions have been investigated by plating. Using this method, Conn (1917) found that the large spore-forming rods whose colonies are conspicuous on plates do not vary much in numbers and thus concluded that they are largely inactive in the soil. On the other hand he claimed the importance of the large numbers of short rod forms which comprise 40 to 75 per cent. of the bacterial population, but which, owing to their slow growth on plates, have at present scarcely received the attention they deserve.

The second method has been to observe the bacterial activity in the soil itself by direct microscopical examination. The chief difficulty of this method lies in the technique of staining the organisms in smears of the soil, since the humic acid bodies readily take up the basic dyes commonly used for bacteria. Conn (1918), however, attempted the method, using bengal rose, and Winogradsky (1925) has further developed it by using erythrosin, which under favourable conditions gives a good differentiation of the organisms. The direct examination of soil indicates that the various biochemical changes in it are each brought about by one or, at most, by a few types of organisms. Thus Winogradsky (1925) finds that in unmanured soil the only organisms sufficiently numerous to attract attention are micrococci and short sporeless rods, which occur in colonies on the soil colloids. If a small amount of peptone be added to the soil, spore-forming bacilli, previously too scarce to be found, suddenly increase, so that in a few hours their numbers preponderate. After 30 hours the special chemical change they have brought about appears to be completed, for they revert to the spore condition. The addition of urea brings about the increase of two short rod forms, while in the presence of starch a bacillus and an actinomycete multiply. The bacteria involved in these sudden increases exist not as colonies but as separate cells floating in the soil solution. In any but a poor unmanured soil, therefore, we may picture the bacterial population as being in a state of fluctuating change, one group after another coming into prominence in accordance with soil conditions and the type of food supply existing at the time.

Quantitative Study of the Soil Bacteria.

The direct examination of soil thus indicates that under laboratory conditions rapid changes both in the numbers and composition of the bacterial population can take place. In the field, where the presence of varied sources of food supply enables many bacterial processes to take place simultaneously, the changes in bacterial flora will be correspondingly complicated.

In estimating the bacterial numbers in the field the uneven distribution of micro-organisms throughout the soil is a source of difficulty. This can be met by multiplying the number of samples tested, by measuring and making allowance for the variance in the population over the plot of soil, and by comparing the changes in bacterial numbers that occur simultaneously in different parts of the plot. No result can be considered of value unless such precautions have been adequately taken.

At first sight it might appear that a direct method of counting bacteria in soil preparations stained on the slide would not be difficult. Attempts to do this, however, have met with difficulty in obtaining a perfectly even film of soil on the slide, a condition essential in order that the microscope fields counted may give true representative samples of the population. An accurate method of direct counting would be most valuable, but such a method will always be subject to the disadvantage that the microscopic appearance of the cells can alone be used to identify the bacteria involved in a change of numbers. Such methods must always be supplemented by a plating method that renders possible the isolation and study of the organisms that appear to be of importance.

Estimates of bacterial numbers are usually obtained from platings of a highly diluted suspension of soil on a gelatin or agar medium. Gelatin platings have been found useful for distinguishing different organisms owing to the more distinctive colonies formed on this medium. The abundance of soil organisms that liquefy gelatin renders this medium of less value for accurate quantitative work. Many agar media suffer from a corresponding disadvantage in that they encourage certain organisms to form rapidly spreading colonies. But this difficulty can be mitigated by a proper selection of the nutrient constituents (Thornton, 1922), and when a careful technique is employed very good agreement can be obtained between parallel counts made simultaneously from one sample of soil (Fisher, Thornton and Mackenzie, 1922). Such a method thus enables changes in bacterial numbers in soil to be followed. It suffers from the serious limitation that no medium will allow all soil bacteria to develop colonies, since some groups require special media. A plate count does not, therefore, give the *total* numbers of bacteria present but selects certain groups.

Nevertheless, a large mass of data has been collected as to the effect on bacterial numbers of various soil conditions, and of climate and season.

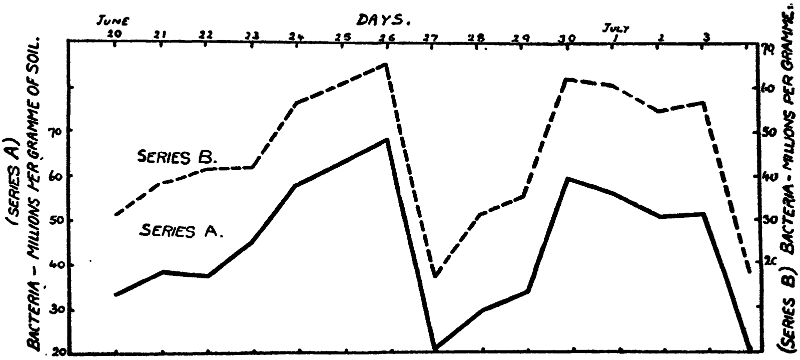

FIG. 1. Daily changes in bacterial numbers in field soil: counts from two series of soil samples taken six feet apart. (After Cutler, from Russell and others, 1923.)

Such counts have usually been made at seasonal or fortnightly intervals, but it is now known that bacterial numbers in the soil vary at much shorter intervals. The changing numbers of bacteria from day to day in a plot of ground on Barnfield, Rothamsted, have been followed during a whole year by means of plate counts. A typical sample of the fluctuations found is shown in Fig. 1, which shows that they take place simultaneously in two separate parts of the plot tested. More recently it has been found that similar fluctuations occur during a period of a few hours.

The chemical and physical conditions of the soil over such short periods seem to be too constant to explain these fluctuations. There is evidence that they are related to fluctuations in numbers of active protozoa. The influence of protozoa on the bacteria in soil was suggested by the effects of partial sterilization. Russell and Hutchinson (1909, 1913) found that if soil were heated with steam, or treated with a volatile antiseptic such as toluene, the bacteria decreased at first and then rose to high numbers and activity, which were maintained for a considerable period. If a little untreated soil was introduced into such partially sterilized soil the number of bacteria was depressed. Thus a limiting factor was introduced with the untreated soil. It was thought that this limiting factor might be protozoa, and these were searched for. Ciliates and amœbæ were found in untreated soil, but were apparently killed by partial sterilization. As a result of this work the protozoan fauna of the soil has been carefully studied, and soils from nearly every part of the world have been searched for these organisms. An extensive fauna of flagellates, ciliates and rhizopods has been found, the species being mostly of world-wide distribution. For full description of this work the reader is referred to Sandon (1927).

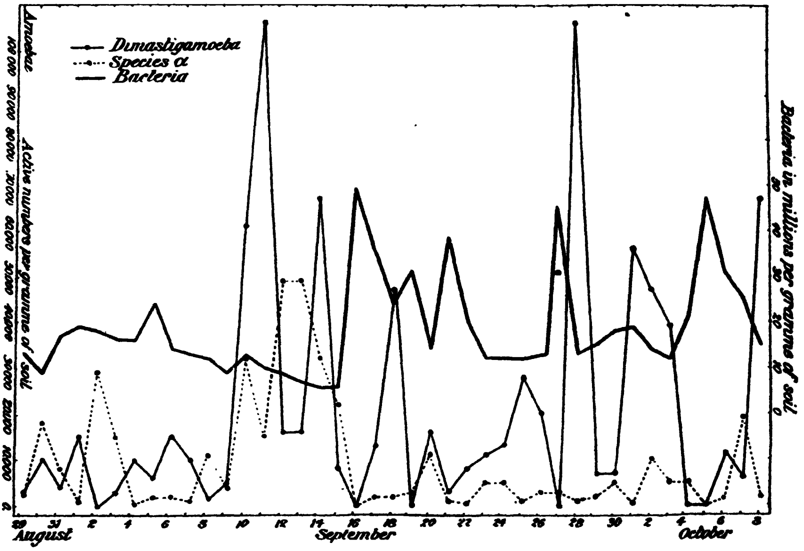

FIG. 2. Daily changes in numbers of bacteria and of active amœbæ in field soil. (From Cutler, Crump and Sandon, 1922, by permission.)

The relationships between protozoa and bacteria in field soil were studied by Cutler, Crump and Sandon (1922), who counted the active and encysted protozoa, belonging to six species, in samples taken from Barnfield, Rothamsted, on 365 consecutive days, and compared their numbers with plate counts of the bacteria. Large changes in numbers of protozoa occurred from day to day and a significant inverse relationship was found to exist between the fluctuations in numbers of bacteria and of active amœbæ (Fig. 2). It is tempting to suppose that the changing numbers of bacteria found at shorter than daily intervals are also connected with protozoan population, but at present the technique of counting the latter does not permit of more frequent than daily counts.

There is some evidence that the bacterial numbers follow fluctuations

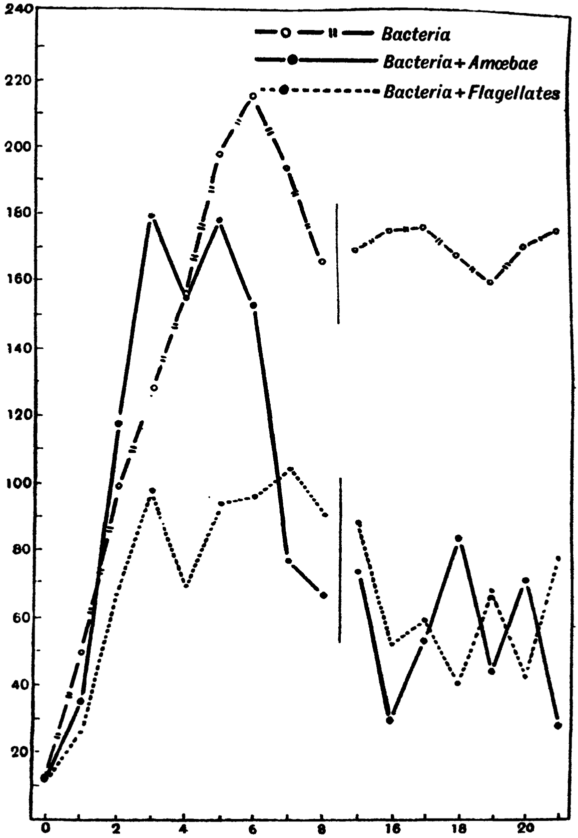

FIG. 3. Bacterial numbers in sterilized soil inoculated with bacteria alone, and with bacteria and protozoa. (From Cutler, *Annals of Applied Biology*, by permission of the Editors.)

primarily induced by the amœbæ and not vice versa, since Cutler (1923) found that if sterilized soil were infected with a population of three species of bacteria alone these increased to a high level and thereafter maintained constant numbers, while, if bacteria and amœbæ were added to such soil, the former were soon kept down to low numbers which showed well-marked daily fluctuations (Fig. 3). On the other hand, it is known that the numbers of, for example, *Bacillus radicicola* when grown in pure culture in soil do in fact fluctuate in a similar manner owing to the existence of a period of multiple fission in the life-cycle (Thornton and Gangulee, 1926). If other soil bacteria possess this feature, it is therefore possible that the numbers of amœbæ may be affected by alternate plenty and scarcity of food supply. The numbers of active soil bacteria and protozoa show seasonal changes that are different in nature from daily fluctuations in that they do not involve a change in relative numbers of bacteria and protozoa, both groups showing a spring and an autumn rise.

The soil also contains a large population of fungi and of algæ whose activity in bringing about changes in the soil may be of great importance (see Russell and others, 1923). The extent to which these groups affect the numbers and activities of the soil bacteria cannot at present be estimated for want of adequate counting methods. The bacteria cannot, however, be considered alone but must be regarded as members of the soil population whose composition is frequently fluctuating.

The Influence of Physical and Chemical Factors on the Soil Bacteria.

When we consider the action of soil conditions on the bacteria, the existence of a complex soil population must be borne in mind. Any change in soil conditions is liable to disturb the equilibrium existing between the competing groups, and may thus produce unexpected results. Thus in arable soil it is usually difficult to detect any relationship between changes in temperature and bacterial numbers (Cutler, Crump and Sandon, 1922). Indeed the freezing of soil may cause a marked increase (Conn, 1914). These results are perhaps due to the active protozoa which are also benefited by temperatures favourable to bacterial growth, but more easily inhibited by temperature extremes. Russell and Hutchinson (1913) found that, under laboratory conditions, a direct relationship between temperature and bacterial numbers existed in soil whose population had been simplified by partial sterilization, though the relationship was masked in untreated soil. Similarly no relationship between daily changes in bacterial numbers and soil moisture can be detected in field soil (Cutler, Crump and Sandon, 1922), though under laboratory conditions large differences in moisture do result in corresponding degrees of bacterial activity (Greaves and Carter, 1920).

The reaction of the soil at its extreme ranges produces marked effects on bacterial activity. Its acidity may increase until the bacterial decomposition of plant remains ceases and a layer of peat is formed. The degree of acidity that is toxic varies greatly with different bacteria, *Azotobacter* and the nitrifying organisms being especially intolerant of acid conditions. The effect of salts on soil bacterial activities has a special importance in dry regions where the problem of soil alkali is often serious. Bacteria are commonly stimulated by traces of salts that are toxic in higher concentrations. This is especially the case with arsenic (Greaves, 1916) and manganese, though the cause of this stimulation is not understood.

The electro-negative ion plays an important part in the toxic action of salts, chlorides being especially harmful. Salts appear to act on the bacteria partly owing to the osmotic changes they produce and partly owing to the specific action of their ions on the bacterial protoplasm (Greaves and Lund, 1921). Changes in the electrical conductivity of bacterial suspensions in the presence of various salts suggests that these affect the permeability of the cell walls (Shearer, 1919). A definite 'antagonism' has been found to exist between various salts, so that two salts, each toxic to soil bacteria, may be less harmful when both are present. A fuller knowledge of what constitutes a well 'balanced' soil solution may thus enable a harmful excess of one salt to be corrected. The presence of potassium and phosphorus in the soil solution is necessary owing to their importance in the composition of the bacterial cell. Since the quantity of soluble phosphates in soil is often a factor limiting the growth of bacteria their addition to soil is generally followed by increased bacterial activity (Fred, 1916).

The amount and nature of the energy material in the soil, and especially the ratio of carbon to nitrogen in it, produces an important effect on the size, composition and activities of the population. Where nitrogenous organic compounds afford the main supply of energy material, the bacteria will release in the form of ammonia such nitrogen as is surplus to their requirements. This ammonia is usually oxidized to nitrate.

Where the main source of energy is a carbohydrate, the bacteria must obtain the nitrogen that they require from an outside source, and so tend to assimilate the ammonia and nitrate and to deplete the soil of these compounds.

Finally, when the soil has become exhausted of its available nitrogen compounds, any further supply of carbohydrate can be utilized only by bacteria that obtain nitrogen from the atmosphere. The process of nitrogen fixation will then become predominant.

The Activity of Bacteria in Soil.

THE DECOMPOSITION OF PLANT REMAINS.

In the soil nearly the whole of the material utilized by bacteria as a source of energy is derived from green plants, and consists of non-nitrogenous compounds, mainly carbohydrates and their derivatives, and of nitrogenous compounds chiefly derived from proteins. When plant remains are added to the soil their disintegration begins with the decomposition of the pectin forming the middle lamellæ of the cell walls. A number of organisms can attack pectin, especially the *Amylobacter* group *B. mesentericus*. A considerable number of bacteria causing plant disease also attack the pectin layer. The separation of individual plant cells by the bacterial decomposition of the pectin is of commercial importance in the retting of flax.

A large number of soil organisms can attack starches and hexoses contained in plant remains, but few appear capable of attacking hemicelluloses. Sawamura (1902) found that several well-known bacteria could decompose mannan, araban and galactan. Dupont (1902) found that xylan could be attacked by an aerobic thermophilic organism resembling *B. mesentericus* (Flügge), while Stoklasa (1898) found that another aerobic soil organism, *Bact. ellenbachensis* (Stutzer), could decompose this compound. It appears, however, that pentoses are decomposed less readily under the aerobic conditions that obtain in normal soil, than under the warmer, anaerobic conditions of the manure heap. Thus Sjollema and Ruyter de Wild (1908) have found that cow dung left under aerobic conditions for two months at room temperature lost only 13 per cent. of its pentosans, whereas under anaerobic conditions at 35° C. it lost 46·5 per cent. in the same period. This may perhaps explain why pentosans and pentoses are widely distributed even in arable soils, where hexoses do not accumulate in measurable quantities.

The large bulk of the remains of plants consists of cellulose and lignin. The earlier investigations into the biochemical decomposition of cellulose were carried out under conditions of deficient oxygen supply. Omelianski (1895) isolated two organisms, *B. methanigenes* (Lehmann and Neumann) and *B. fossicularum* (Lehmann and Neumann). These organisms were both found to attack cellulose, the former producing fatty acids, methane and CO_2 and the latter fatty acids, hydrogen gas and CO_2. Khouvine (1923) described another organism, *B. cellulosæ dissolvens*, which resembles Omelianski's organisms but has oval spores. The products recovered from cellulose fermentation include acetic and *n*-butyric acid, ethyl alcohol, CO_2 and hydrogen. Pure cultures of these three organisms do not seem to have been obtained, but the predominance of each organism in the cultures used was such as to justify the assumption that it was the active agent.[1] The nature of the fermentation processes produced makes it likely that organisms of this type are active in decomposing cellulose in ponds and also in water-logged soils. Harrison and Aiyer (1914), when studying the swamp soils of rice fields, found that when a green manure was ploughed in, methane, hydrogen and CO_2 were evolved in the body of the soil. At the surface, however, methane and hydrogen were not found and their disappearance was traced to a film of organisms having the power of oxidizing these gases. The green manure was only beneficial to the crop while this film was working. Bacteria capable of deriving energy by oxidizing hydrogen gas have been isolated by Kaserer (1906) and by Nabokich and Lebedeff (1907), while Söhngen (1905) has isolated an organism that will oxidize methane.

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[1] *B. methanigenes* Lehmann and Neumann, *B. fossicularum* Lehmann and Neumann, and *B. cellulosæ dissolvens* Khouvine, are closely similar organisms. They exist normally as very slender rods often bent or curved, bearing terminal spores, which in the last-named species are oval and in the first two round. The dimensions of the cells differ slightly in the three species. *B. methanigenes*: Rods, 5 to 15 × 0·4μ; spores, 1μ diameter. *B. fossicularum*: Rods, 4 to 15 × 0·5μ; spores, 1·5μ diameter. *B. cellulosæ dissolvens*: Rods, 2·5 to 12·5 × 0·5μ; spores, 2 × 2·5μ. The organisms will not grow on ordinary laboratory media. *B. cellulosæ dissolvens* occurs in the human intestine and also in soil.

The anaerobic fermentation of cellulose at high temperatures has been studied by several workers on account of its importance in manure and compost heaps and in stacks. Organisms have been described by Kroulik (1912–13), by Langwell and Hind (1923), and by Viljöen, Fred, and Peterson (1926), which are said to operate at temperatures of about 60° C. It is uncertain, however, whether any of these organisms were obtained in a pure state.

In normal arable soils, cellulose decomposes under aerobic conditions. An interesting organism known to attack cellulose in undoubtedly pure culture was isolated by Hutchinson and Clayton (1919) and named *Spirochæta cytophaga* (Fig. 4). Organisms probably identical with it have been described by Van Iterson (1903) and by Gescher (1922). This organism occurs in young cultures in the form of tapering rods that stain with difficulty and are flexible, curving into S and C shapes. The rods develop deeply staining granules and readily pass into a spherical form that has been termed the ‘ sporoid ’ stage. The sporoids are killed by a temperature of 60° C. and are thus not true resistant spores, but are perhaps homologous to similar bodies described in the *Spirochætoidea*. In most cultures all stages of the life-cycle can be found together. This organism is very specific in its action. Cellulose is the only carbohydrate which it attacks; many others, such as dextrose and maltose, being actually toxic to it. It prefers an inorganic source of nitrogen and its growth is inhibited by peptone above 0·25 per cent. Its sources of food may be less limited under natural conditions, but until this is demonstrated it is difficult to suppose that it can attack plant remains until these have lost the most of the soluble and easily decomposed organic matter. Another aerobic organism has been studied in pure culture by Gray and Chalmers (1924) and named *Microspira agar-liquefaciens*[1] (Fig. 5). It attacks filter

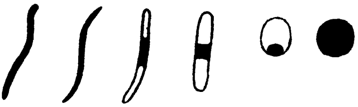

FIG. 4. *Spirochæta cytophaga*. Mean length of filamentous cells, 3μ. (From Russell and others, 1923.)

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[1] *Microspira agar-liquefaciens* Gray and Chalmers. Short curved rods, 2 × 0·5 to 0·7μ, bearing single terminal flagella. Gram-negative. Spores unknown. On nutrient agar at 25° C., the growth is white and occupies a depressed area of liquefied agar. Colonies are surrounded by an area of semi-liquefied agar. On gelatin, there is very slight growth and no liquefaction. Acid is produced from dextrose, lactose and maltose, but not from sucrose or glycerin. No gas is formed. There is pronounced diastase formation on starch agar. On nitrate broth a slight formation of nitrite occurs. In mineral salts solution containing filter paper, the latter is rapidly attacked in the presence of air.

paper rapidly and also liquefies agar. Unlike the spirochæte it can live in ordinary media. The presence of small amounts of xylose and lignin increase the amount of cellulose decomposed by it, a fact of great interest in view of the common association of these compounds in nature. Kellerman, McBeth and others (1913) have isolated about 50 species of aerobic bacteria claimed to be capable of attacking cellulose. Later workers examining cultures of these organisms have sometimes found them incapable of attacking cellulose (Pringsheim and Lichtenstein, 1924). Bradley and Rettger (1927) have recently investigated eight of these organisms and have themselves isolated seven other strains. They find that though their activity was very slight when in a solution of inorganic salts and filter paper, their cellulose-decomposing power was much increased by the presence of organic matter such as peptone or digested casein. This effect is analogous to the stimulating action of xylose and lignin found by Gray and Chalmers. Most of the organisms isolated by these American workers are short, non-sporing rods. Similar organisms, claimed to attack cellulose, are described by Sack (1924). Recently a number of bacteria have been isolated from British soil by Kalnins and found to attack cellulose. Most of these are also small, non-sporing rods. It is likely, therefore, that this type of organism is of chief importance in the decomposition of cellulose in arable soil.

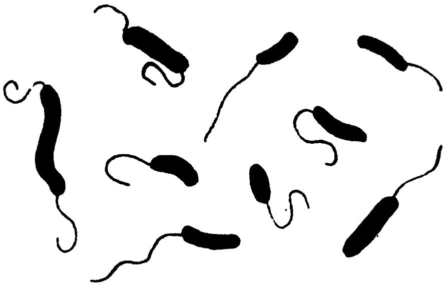

FIG. 5. *Microspira agar-liquefaciens.* Mean length, 2μ. (From Gray and Chalmers, *Annals of Applied Biology*, by permission of the Editors.)

Groenewege (1920) isolated three organisms which he named *Bac. cellaresolvens* α, β, γ, and which he claims can decompose cellulose when acting together with certain denitrifying bacteria resembling *Bact. fluorescens*, although in pure culture they do not possess this power. The claim that micro-organisms can produce certain biochemical changes only in association with other types must, if substantiated, greatly affect the investigation of such processes in the soil.

In addition to bacteria a number of actinomycetes occurring in soil appear capable of attacking cellulose (Krainsky, 1914), while this power is also possessed by a large number of fungi (for references see Russell and others, 1923, p. 133). The relative importance of bacteria and fungi in soil in this as in other biochemical processes is difficult to estimate at present owing to the lack of reliable quantitative methods.

The decomposition of lignin in soil, although of great importance, is still very obscure, nor is it certain to what extent it is due to biological agency. When plant remains decompose in soil, brown substances collectively known as humus are formed. This humus produces effects on the condition of the soil that are of the greatest agricultural importance. At present, however, neither the chemistry nor the biological causes of its formation are understood. The bacteria so far studied in pure culture do not produce humus. There is chemical evidence, however, that some at least of the humic bodies are derived from lignin, while others may be derived from the mycelium of certain fungi.

In addition to carbohydrates and their derivatives there are certain other non-nitrogenous organic compounds introduced into the soil with plant remains and farmyard manure. Amongst these are the aromatic compounds, such as phenol, which is formed in the bacterial decomposition of protein and which occurs in appreciable amounts in liquid manure. The disappearance of aromatic compounds from soil has a special interest, because the cresols, phenol and naphthalene are used to destroy pests in greenhouse soils. A number of organisms are known that can attack phenol, cresol and naphthalene (Gray and Thornton, 1927). These organisms belong to a variety of morphological types, including mycobacteria, micrococci, sporing and non-sporing rods. Some of them exhibit a series of remarkable cell forms when growing in culture (Fig. 6). A few can also attack toluene. Organisms capable of attacking phenol have also been described by Wagner (1914) and by Fowler, Ardern and Lockett (1910).

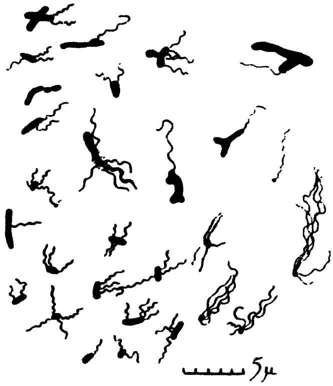

FIG. 6. *Mycoplana dimorpha.* (From Gray and Thornton, 1927.)

THE NITROGEN CYCLE.

The second main group of products left in the soil by plants are the nitrogen-containing compounds, the proteins, amino-acids, &c. Plant remains are not the only source of such compounds in the soil, there being also the dead cells of other soil organisms such as worms, insects, protozoa and algæ. Bacteria are able to utilize many organic nitrogen compounds, deriving energy therefrom, as can be exemplified by the oxidation of a simple amino-acid.

$$NH_2 - CH_2 - COOH + 3O = 2CO_2 + H_2O + NH_3 + 152 \text{ Cal.}$$

It will be seen that in the acquirement of energy in this way, ammonia is set free as a by-product. Most common ammonia-producing organisms can also derive their energy from sugars and related compounds so that the formation of ammonia is reduced by the presence of such alternative sources of energy (Doryland, 1916). There is little doubt that the breakdown of protein and the release of the ammonia is a complicated process involving the activity of a variety of different organisms. Large numbers of soil bacteria and fungi can produce ammonia from peptone in culture media, but it is not yet known which of them are of importance as ammonifiers in the soil itself.

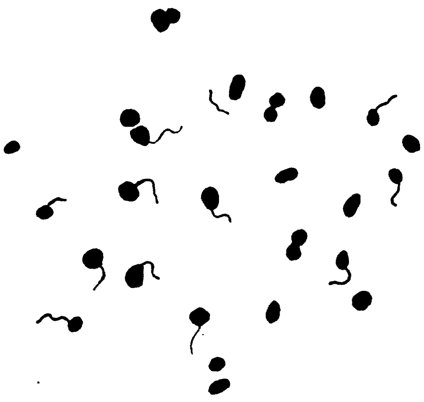

FIG. 7. *Nitrosomonas* Winogradsky. Average length of cell, 1·5μ.

The ammonia produced in the soil does not normally accumulate, though considerable amounts sometimes collect in forest soils. In normal arable soil it is oxidized successively to nitrite and nitrate. Our knowledge of this process is mainly due to the work of Winogradsky (1890), whose results have in general been confirmed by Bonazzi (1919, 1921, 1923) and Gibbs (1919). Winogradsky isolated two organisms capable of oxidizing ammonia to nitrite and gave them the generic names *Nitrosomonas* (Fig. 7) and *Nitrosococcus*. It has been suggested that the latter type is a stage in the life history of *Nitrosomonas*, which is the better known organism.[1] It is intolerant of an artificial medium containing organic matter, but is able to grow in a culture solution containing an ammonium salt, mineral salts, but no organic carbon. Under such conditions it is clear that it must synthesize its protein by taking up carbon from CO_2 or bicarbonate in the solution. Since growth takes place in the dark the possibility of photo-synthesis is excluded. The organisms must therefore find in the solution a source of energy sufficient to enable them to assimilate CO_2. The only energy source being the ammonium salt, it follows that the bacteria obtain energy by oxidizing ammonia to nitrite :

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[1] *Nitrosomonas europea* Winogradsky. Oval cells, 1·2 to 1·8 × 0·9 to 1μ. Frequently form zooglœal masses. In the motile condition bear single terminal flagella. No spores known. Will not grow on ordinary laboratory media. Utilizes HN_4 salts as its source of energy and CO_2 as its source of carbon. Aerobic.

$$(NH_4)_2CO_3 + 3O_2 = 2HNO_2 + CO_2 + 3H_2O + 148 \text{ Cals.}$$

Winogradsky (1890) and Coleman (1908) found a definite relationship between the amount of ammonia oxidized and the carbon assimilated. The course of oxidation of the ammonia to nitrite is not known. It has been suggested that hydroxylamine may be formed, but this compound has not been identified in cultures. The nitrite formed does not accumulate in the soil as it is rapidly oxidized to nitrate. Winogradsky isolated a short, rod-shaped organism able to bring about this change, which he named *Nitrobacter*.[1] Like *Nitrosomonas* it is able to live in solutions of mineral salts, assimilating inorganic carbon. It obtains the necessary energy to do this by the oxidation of nitrite to nitrate.

The activity of both *Nitrosomonas* and *Nitrobacter* in artificial culture media is inhibited by soluble organic matter. It appears, however, that under natural conditions the oxidation of ammonia to nitrate can occur in an environment rich in organic compounds. For example, it takes place rapidly in richly manured soils and in the purification of sewage. The conditions with regard to aeration and the removal of the by-products of metabolism are very different in culture media and under natural conditions. Bonazzi (1919) has found that nitrification in a culture solution was greatly increased by shaking. It is, however, possible that other organisms, more tolerant of organic matter, may contribute to the oxidation of ammonia in nature. Sack (1925) has described four bacteria which he claims can oxidize nitrite and are not sensitive to the presence of organic matter. His results, however, require confirmation. Both *Nitrosomonas* and *Nitrobacter* are intolerant of acidity. The optimum reaction for the oxidation of ammonia is pH 8·4 to 8·8, while that for the oxidation of nitrite is pH 8·4 to 9·3. Here again, however, it is probable that the conditions in nature are less restricted, since nitrification can occur in soil having a reaction of pH 4·0. *Nitrosomonas* requires the presence of an available base to neutralize the nitrous acid formed. Calcium or magnesium carbonate usually acts in this way, but it has been found that, in a liquid medium, insoluble calcium phosphate can be utilized, a solution of the phosphate being brought about (Hopkins and Whiting, 1916).

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[1] *Nitrobacter* Winogradsky. Rod-shaped cells, 1 to 1·5 × 0·3 to 0·7μ. Non-motile. Gram-negative. No spores known. Will not grow on ordinary laboratory media. Utilizes nitrite as its source of energy and CO_2 as its source of carbon. Aerobic.

In the cases above described, the bacteria usually obtain energy through an oxidation process. In arable soils the soil atmosphere commonly supplies the necessary oxygen, but in water-logged soils or in the presence of large excess of organic matter, anaerobic bacteria, which obtain their oxygen from oxidized compounds, may develop. The most important source of oxygen under these conditions, is nitrate. A large number of bacteria are able to reduce nitrate to nitrite; many can reduce nitrate to ammonia, while a few can reduce nitrate to nitrogen gas. In swamp soils in Japan, nitrate of soda often depresses the yield of crops, probably owing to its reduction to poisonous nitrites.

About 10 per cent. of the dry weight of bacteria consists of nitrogen. This nitrogen is sometimes obtained from organic nitrogen compounds, but a large number of common soil organisms can assimilate nitrogen from ammonia or nitrate. When the supply of available carbohydrate material is sufficient, the organisms deriving energy from this source will increase greatly and may assimilate sufficient nitrate to produce nitrogen starvation in the crop. Such an effect is produced by ploughing in fresh straw (Martin, 1925).

It is possible that soil algæ, in addition to fungi and bacteria, may be of importance in nitrate assimilation. Algæ are active not only on the surface but also within the soil, where their energy is presumably derived from organic matter. The assimilation of nitrates by micro-organisms greatly lessens the losses of nitrogen from soil by leaching, and, at times when the field is not growing a crop, the process is of value in preserving nitrogen which will later be reconverted into nitrate. In this storage of nitrogen it is probable that protozoa play their part.

In addition to the micro-organisms that assimilate nitrogen compounds there is a smaller but very interesting group of bacteria capable of taking in nitrogen gas and building it up into protein. The first nitrogen-fixing organism was discovered by Winogradsky (1895), who named it *Clostridium pasteurianum* (Fig. 8). It is a rod, forming central endospores, and belongs to the group studied by Bredeman under the name of *Amylobacter*.[1] The organism in pure culture was found to be anaerobic, and Winogradsky (1924) has recently found that this type of rod increases in water-logged soil to which excess of sugar is added. Mixed cultures of the *Clostridium* and aerobic bacteria can grow in the presence of air, the latter presumably

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[1] *Clostridium pasteurianum* Winogradsky is probably a variety of *Clostridium butyricum* Prazmowski. The cells are straight cylindrical rods, 0·75 to 1·0μ × 3·0 to 10·0μ, usually occurring singly or in pairs. They bear peritrichous flagella, and are Gram-positive. Glycogen is stored in the protoplasm. The spores are oval, 1·5 × 2μ, and are produced subcentrally. The spore mother-cell becomes greatly thickened in the centre and its protoplasm becomes granular, staining with difficulty with aniline dyes. Gelatin not liquefied. Dextrose agar slope: (anaerobic) grey, flat, moist spreading with irregular lacerate margin. Dextrose broth: turbid. Litmus milk: acid, coagulation, with abundant gas formation. Nitrates reduced to nitrites and ammonia. Acids, gas and alcohols formed from dextrose, maltose, lactose, sucrose, and starch. The organism is normally anaerobic.

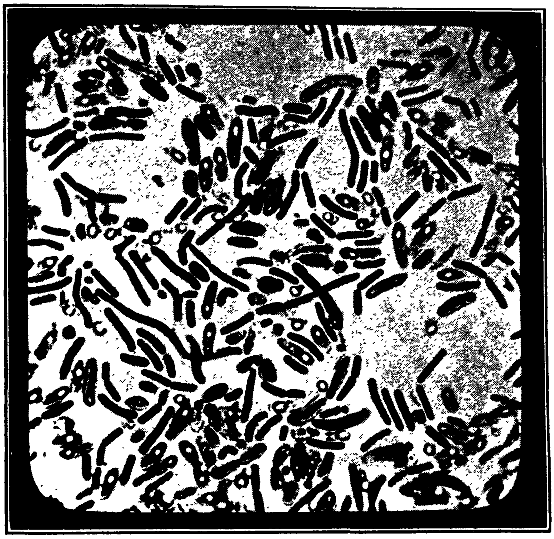

FIG. 8. *Clostridium pasteurianum* Winogradsky. Young culture, mean width of cell 1μ.

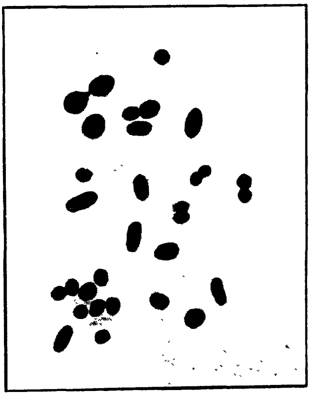

FIG. 9. *Azotobacter chroococcum* Beijerinck. Young agar culture, cells 2 to 5μ long.

[*To face p. 102.*

removing oxygen and creating an anaerobic environment for the former (Oméliansky and Solounskoff, 1915).

There is evidence, however, that in well aerated soil the truly aerobic nitrogen-fixing organisms are of chief importance. Of these the first discovered and best known is *Azotobacter* (Beijerinck, 1901) of which several species have been described.[1] In its commonest form *Azotobacter* occurs as ovoid cells, but (Fig. 9) Löhnis and Smith (1916) have described a complicated series of cell forms that develop in cultures of the organism. *Azotobacter* is of world-wide distribution in soil, and it is known from direct microscopical examination that it can multiply rapidly in soil supplied with energy material (Winogradsky, 1925). It is thus probably an active agent in soil under aerobic conditions. It is very sensitive to soil acidity, and does not grow in soil having a hydrogen-ion concentration of less than pH 6·0. The cells of *Azotobacter* are very rich in phosphorus, their ash containing about 60 per cent. of phosphoric acid, and they require to be well supplied with phosphate to ensure maximum growth. Christensen has indeed found soils where lack of phosphate was the limiting factor for *Azotobacter*.

In addition to *Azotobacter* a number of other aerobic organisms that are said to fix nitrogen have been described. Amongst these are large spore-forming motile rods described by Bondorff (1918) and a *Spirillum* described by Beijerinck (1925). There is also the possibility that *Bacillus radicicola* (Beijerinck) may be an important nitrogen fixer in soil, where it occurs in considerable numbers.

Azotobacter and probably the other nitrogen-fixing bacteria will assimilate nitrogen compounds where these are available in preference to fixing free nitrogen. Addition of highly nitrogenous substances to soil consequently reduces or stops nitrogen fixation.

The process by which nitrogen is fixed is at present unknown. In young cultures of *Azotobacter* a large percentage of the nitrogen exists in the form of amino-acids, suggesting that the nitrogen passes through the amino stage during protein synthesis (Waynick and Woodhouse, 1918). This perhaps implies that the nitrogen is initially combined with hydrogen and not with oxygen. The bacteria probably obtain hydrogen from carbohydrates and related substances. It has been claimed, on thermodynamical grounds, that the combination of nitrogen and hydrogen requires no expenditure but causes a release of energy (Burk, 1927). In culture solutions, however, about 100 gms. of dextrose are utilized by

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[1] *Azotobacter chroococcum* Beijerinck. In young cultures the cells are usually oval, 2 to 4μ × 3 to 6μ, with mucilaginous capsules. They frequently occur in pairs. The cell content is often vacuolated and contains granules. A few motile cells occur, bearing single polar flagella. In older cultures sarcina-like forms are frequent. The organism is aerobic, and on liquid media it forms a slimy pellicle. In older cultures a dark brown pigment is produced. Gelatin colonies: small, circular, yellow granular. No liquefaction. Only slight growth on gelatin stab and no growth in broth. On potato: glossy slimy or wrinkled growth, chocolate brown. A large number of carbon compounds are oxidized to CO_2 and H_2O.

Azotobacter in the fixation of 1 gm. of nitrogen, though the figure is very variable. The large amount of dextrose required suggests that this is being consumed in order to supply energy for the building up of protein. A large number of compounds, including sugars, alcohols and organic acids, can be utilized by *Azotobacter* as sources of energy. Of special interest is the fact that the decomposition products of cellulose form available energy material for nitrogen-fixing bacteria. Thus Hutchinson and Clayton (1919) found that the products of the action of *Spirochæta cytophaga* could be so used.

In the soil the efficiency of fixation, i.e. amount of nitrogen combined per gramme of carbohydrate consumed, is very much greater than in culture solution. This is perhaps due to the harmful accumulation of the by-products of metabolism, under the latter conditions. Koch and Seydel (1912) have found that, in solution cultures, the efficiency of fixation by *Azotobacter* is high when the culture is only a few days old, but falls off with age of culture along a curve that strongly suggests the effect of accumulating inhibitory substances. Golding (1905) found that, with *Bacillus radicicola,* nitrogen fixation came rapidly to a stop when cultures were grown in flasks of liquid medium, but that a considerable fixation could be obtained if the products of their metabolism were continuously removed by means of a filtering device. This need for the removal of by-products perhaps explains the cases in which it has been found that nitrogen fixation by bacteria is more efficient when these are growing in association with other organisms. Thus Beijerinck and Van Delden (1902) found that impure cultures of *Azotobacter* fixed more nitrogen than pure cultures, while Hansawa (1914) found that fixation was more active when two strains of *Azotobacter* were grown together than when they were separated. A special case has been investigated by Nazir (1923) and by Cutler and Bal (1926), who found that nitrogen fixation by *Azotobacter* was stimulated by the presence of protozoa, which fed on the *Azotobacter,* thus preventing the accumulation of dead and senescent cells.

Nitrogen-fixing bacteria are also found associated with green plants, which, in addition to removing the by-products of metabolism, also supply the bacteria with the necessary carbon compounds. The simplest of such cases is their occurrence together with green algæ, investigated by Kossowitsch (1894) and others.

The frequent association of nitrogen-fixing bacteria and algæ has indeed given rise to statements that the latter can fix nitrogen, but Page and Bristol (1923) have found that, with proper care in obtaining bacteria-free cultures of algæ, no such fixation was obtained. On the other hand, Lipman and Teakle (1925) found an appreciable fixation of nitrogen in mixed cultures of *Azotobacter* and *Chlorella* in a liquid medium, devoid of other organic matter than that produced by the algæ.

A special case of symbiosis with green plants is the growth of nitrogen-fixing bacteria within the leaves and roots of higher plants. Of these bacteria by far the best known is *Bacillus radicicola* (Beijerinck, 1895), which forms nodules on the roots of leguminous plants. Such nodules were figured in Gerarde's herbal in 1633 and by Malpighi in 1686, who described them as galls.

The fact that leguminous crops enriched the soil was known since Roman times. The study of the nitrogen requirements of crop plants initiated by Lawes and Gilbert about the middle of the nineteenth century showed that legumes differed from other crops in their independence of combined nitrogen. Hellriegel and Wilfarth's pot experiments connected this with the nodules. They found that if peas were grown in sterile nitrogen-free sand they failed, but that good growth could be produced if a little extract of fertile soil were added. Where the plants failed, their roots were free from nodules, but where they grew, nodules were present. From such experiments it was concluded that the nodules were due to infection by soil organisms and were responsible for the fixation of free nitrogen utilized by the plant. The bacteria were isolated by Beijerinck (1888) and named by him *Bacillus radicicola*.

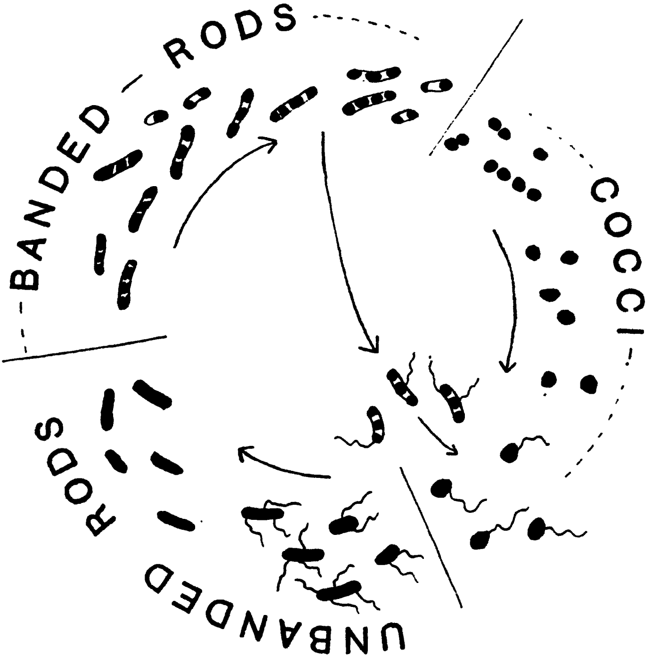

FIG. 10. Life cycle of *B. radicicola*.

The organism exists in several cell forms which have been described by numerous workers. Löhnis and Smith (1916) and Bewley and Hutchinson (1920) described these forms as stages in a definite life-cycle, through which the organism passes in cultures. The existence of a similar cycle in the tissues of the host plant was described by Walling (1922) and its occurrence in the soil by Thornton and Gangulee (1926) (see Fig. 10).

In addition to its life within the tissues of its host plant, *B. radicicola* can spend an active existence in soil when the conditions, especially with regard to the reaction, are favourable to it. It is probable that it reaches the roots of its host by active migration through the soil. Under favourable conditions in the laboratory, it can move through soil at an approximate rate of one inch in 24 hours, and there is evidence that this migration is associated with the appearance of the rounded cells which in liquid cultures are seen to become actively motile (Thornton and Gangulee, 1926). Observations in the field, however, show that under natural conditions the extent to which the bacteria spread is greatly affected by soil and weather conditions.

They enter the roots of their host plant through the unicellular root hairs and in consequence can only infect young roots. A small colony is formed near the tip of the root hair and the thin cell wall is penetrated at this point. The method of penetration is not exactly known, but the fact that the tip of the hair curls over suggests that the wall is softened at the side attacked. Inside the hair the bacteria form a thread consisting of mucilage in which lie numerous minute capsulated rods; this thread grows down the hair and passes through the cortical cells towards the centre of the root (Fig. 11). On reaching a cell wall the thread becomes flattened against it and the bacteria perforate the wall at a spot near the centre of the area of contact. The cells near the infecting thread are stimulated to divide, especially in the deeper layers of the cortex, thus forming a bun-shaped mass—the young nodule. The thread begins to ramify through the deeper nodule tissues and there produces a mass of cells which cease to divide and increase in size. The outer layers, however,

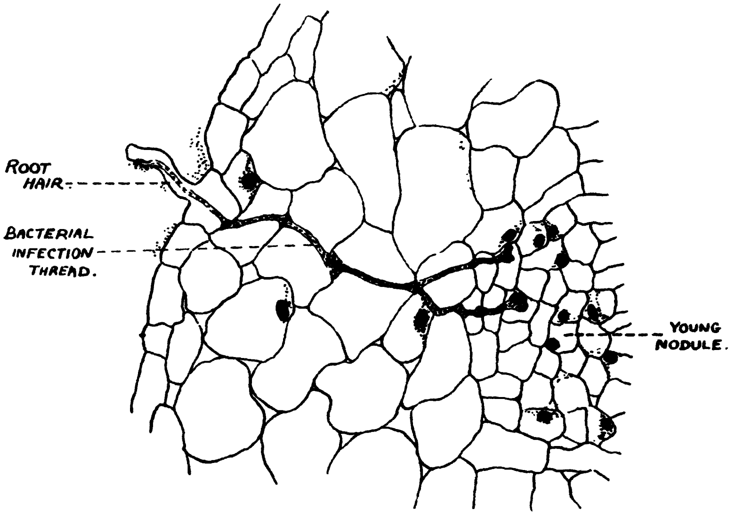

FIG. 11. Part of the root cortex of *Vicia faba*, showing infection by *B. radicicola*.

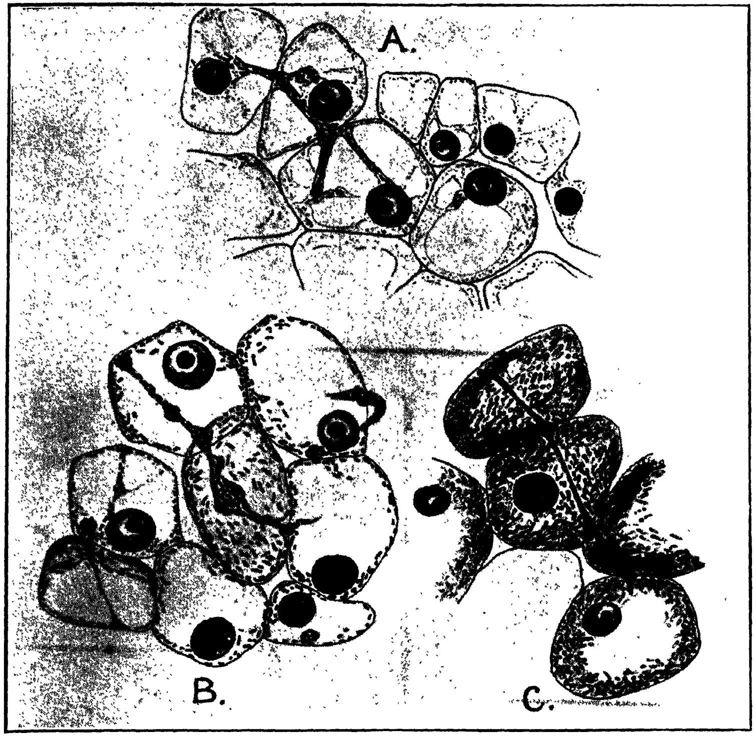

FIG 12. Cells from a nodule of *Vicia faba*, showing the course of infection by *B. radicicola*. A.—Intrusion of the infection thread. B.—Release of the bacteria from the infection thread. C.—Multiplication of the bacteria. (From Brenchley and Thornton, 1925.)

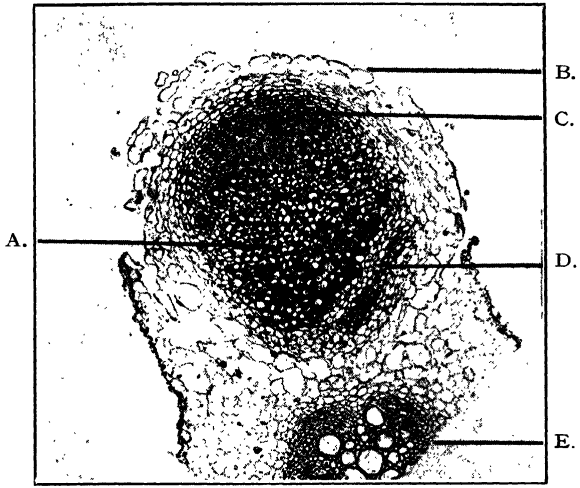

FIG. 13. Section of a nodule of *Vicia faba*. A.—Infected tissue. B.—Nodule cortex. C.—Cap of dividing cells. D.—Vascular strand. E.—Central cylinder of root. (From Brenchley and Thornton, 1925.)

[*To face p. 107.*

remain as a cap of small-cell tissue in a state of rapid division, which continually lengthens the nodule (Fig. 13), while the bacterial threads successively invade the new cells produced. When the infected cells begin to swell, the bacteria are released from the threads and lie in the cytoplasm (Fig. 12). Here they multiply greatly and pass into the banded rod condition, often becoming branched.

At about this stage, vascular strands grow out from the central cylinder of the root and pass up the sides of the nodule tissue (Fig. 13). These strands no doubt perform the double function of supplying the nodule bacteria with carbohydrate energy material and of removing the products of their metabolism. It is possible, in water culture, to induce the formation of nodules in which this vascular supply is deficient. Under such conditions there is no nitrogen fixation, but the bacteria attack and destroy the cells in which they lie (Brenchley and Thornton, 1925). They are thus able to attack the protoplasm of the host but do not do so as long as vascular strands bring an alternative energy source.

The legume bacteria are, however, very specific in their powers of infection. They are divided into physiological varieties, each of which produces nodules on a small group of host plants. Burril and Hansen (1917), by cross inoculation methods, formed eleven groups of nodule bacteria within each of which the host plants are interchangeable. A strain derived from one group of host plants will not readily produce nodules on plants of any other group, though whether it is entirely unable to do so under any conditions is not established. These physiologically distinct strains of the nodule bacteria can also be identified by serological methods, such as agglutination and complement fixation (Klimmer and Krüger, 1914). Stevens (1925) and Wright (1925), however, have found that amongst the nodule bacteria capable of infecting a single host plant several serologically distinct varieties can be identified, which also differ in certain cultural characters, and in one case in their efficiency in increasing the nitrogen content of the host plant.

The idea of supplying legume crops with nodule bacteria, so as to increase their growth, occurred to workers in this field as early as the nineties of last century. After a period of varying success the value of this 'inoculation' is established in certain cases. The fact that given species of legumes are infected by their own specific varieties of nodule bacteria closely concerns the practice. The conditions are different where a legume crop is introduced into a new area, the soil of which does not contain the appropriate variety of nodule organism, and in cases where native or long introduced legumes are grown and find the soil naturally infected with their specific bacteria. In the first case, 'inoculation' may be expected to benefit the crop and may be essential to its successful growth. The cultivation of lucerne (*Medicago sativa*) in England affords an instance of this. Since its introduction about 1630, this crop has been grown fairly extensively in Essex, Kent and the neighbouring counties, where the soil has by now become infected with the lucerne nodule organism.

In the west, middle and north of England, lucerne is seldom grown, and commonly fails when seed is sown without inoculation. It has been found, however, that successful crops can often be grown if the plant is supplied with the proper bacteria, and that in nearly all cases in these areas an increased crop follows inoculation. In the case of legume crops sown in soil already containing their specific organisms, there is usually little or no benefit from inoculation, though the greater development of nodules in the early life of the plant may give it a permanent advantage. Thus when lucerne is sown in a cover crop, it is sometimes benefited by inoculation even in soil already containing lucerne bacteria, because, owing to its more numerous nodules, the young plant need not compete for nitrogen with the stronger cereal.

The Activities of Bacteria in Soil.

THE INFLUENCE OF BACTERIA ON THE SOIL MINERAL CONSTITUENTS.

Soil bacteria, in addition to bringing about the changes in carbon and nitrogen compounds, profoundly affect the mineral constituents of the soil. The sulphur contained in proteins can be released by a variety of common bacteria as H_2S or mercaptans. Hydrogen sulphide can be decomposed by a number of organisms, such as *Beggiatoa,* with the formation of amorphous sulphur. Both hydrogen sulphide and sulphur are utilized as sources of energy by many soil bacteria, which oxidize them to form sulphuric acid. The most remarkable of them is *Thiobacillus thioxidans,*[1] which is able to utilize the energy derived from this reaction to assimilate carbon from carbon dioxide (Waksman and Joffe, 1922). Sulphates in the soil can, on the other hand, be reduced to hydrogen sulphide. Beijerinck (1895) has described an organism *Spirillum desulphuricans* that possesses this power.

Of great agricultural importance are the phosphorus changes in the soil. Nucleoproteid phosphorus occurring in organic compounds in soil is rapidly decomposed by soil bacteria (Koch and Oelsner, 1922) with the formation of organic phosphates. On the other hand, part of the soluble phosphates in soil are assimilated by bacteria, some of which, e.g. *Azotobacter,* have a high content of phosphorus. Bacteria play an important indirect role in changing insoluble basic into soluble acid phosphates by means of the acids produced in their metabolism. These consist mostly of organic fatty acids and carbonic acid. The sulphur-oxidizing bacteria produce sulphuric acid in quantities which have an appreciable effect in the solution of phosphates, and there is evidence that *Nitrosomonas* can utilize tri-calcium phosphate as a base to neutralize the nitrous acid that it produces. The acids produced by micro-organisms in the soil no doubt play an important part in rendering soluble other important plant nutrients, such as potassium, which in most soils is derived from silicates.

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[1] *Thiobacillus thioxidans* Waksman and Joffe. Short rods, $0 \cdot 5 \times 0 \cdot 8\mu$, non-motile. Gram-positive. Aerobic: will not grow on ordinary laboratory media. Inorganic media containing sulphur are suited to it. It derives energy by oxidizing sulphur and thiosulphates and obtains its carbon from CO_2. The optimum reaction for growth is about pH $2 \cdot 5$.

MICROBIOLOGICAL ANALYSIS OF SOILS.

The fundamental importance of bacteria in the production of plant nutrients in soil has led to many attempts to correlate bacterial numbers and activities with soil fertility, and to devise methods of measuring the fertility of a soil by its bacterial activity. Comparisons between the bacterial numbers of soils of known fertility have given varying and uncertain results, as is to be expected in view of the fluctuating equilibrium that exists between the different groups of micro-organisms.

Tests of biochemical activity have usually consisted in placing definite amounts of soil in appropriate culture solutions and estimating, for example, the amount of ammonia or nitrate produced, or the quantity of nitrogen fixed in a given time. Such methods are sometimes made more natural by conducting them in samples of the soil itself instead of in solutions.[1] Under laboratory conditions, however, the environment is very different from that in the field. Indeed, Cutler and Dixon (1927) have shown that when a soil is stored in the laboratory precautions are necessary to prevent a steady falling off in numbers of both bacteria and protozoa. It is indeed likely that such methods do not so much gauge the activity of the soil organisms in the field, as indicate whether the soil in question is of a suitable type for the organisms producing given biochemical changes. Since the soil conditions suitable to the bacterial population on the whole favour the growth of higher plants, an approximate estimate of fertility is often obtained. This use of bacterial activity to test the soil as a medium for growth will perhaps supply a biological index by which a deficiency of important constituents can be detected. Thus Christensen and Larsen (1911) developed a method of estimating the lime requirement of soils by the growth in them of *Azotobacter*, which will not develop in an environment on the acid side of pH 6·5. The rate of nitrate production from ammonium compounds or indirectly from organic nitrogen has also been found to be related to the reaction of the soil (Waksman, 1923). The rate at which cellulose decomposes in soil has been found to be limited by the amount of readily available nitrogen compounds (Waksman and Heukelekian, 1924). The addition of sugar or mannitol to neutral soil causes a multiplication of nitrogen-fixing bacteria which is limited by their need for phosphorus. The destruction of mannitol in neutral soils (Christensen, 1923, Waksman, S. A. and Karunaker, N.) and the production of CO_2 therefrom (Petersen, 1926) have hence been used as tests of the amount of available phosphorus compounds in the soil. Such microbiological tests when more fully developed should thus afford a means of determining directly how much of the phosphorus and nitrogen in a soil is present in a form available to micro-organisms, a question that cannot be answered by chemical analysis. It is probably along such lines that the micro-organisms can best be used to test soil conditions.

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[1] The literature of such tests is extensive and references to it can be found in Waksman's papers in *Soil Science*, 1923–4, and in his *Principles of Soil Microbiology*, 1927.

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CHAPTER VI. BACTERIAL DISEASES OF INSECTS.

BY R. ST. JOHN-BROOKS
(LISTER INSTITUTE, LONDON).

Introduction.

Problems of Control.

FROM the economic standpoint the bacterial diseases of insects may be conveniently divided into two classes—those which affect the larval or imaginal stages of insects of economic value (silkworms, bees) and those tending to originate epizootics among injurious insects or their larvæ. The problem of control, therefore, is, in the two cases, an essentially different one: in the former the aim is directed towards the destruction of the parasite, in the latter to the spread of infection and the production of more virulent strains of the infecting agent.

The classical investigations of Pasteur (1870) on the control of Flacherie among silkworms and the researches of d'Herelle (1914) on the artificial dissemination of locust septicæmia, may be cited as examples of the methods which may be employed in the control of the two different classes of insect diseases under consideration. The solution of the problem in the first group does not present any inherent difficulties and the incidence of various bacterial infections affecting silkworms and bees, for example, have by means of prophylactic measures shown a considerable decrease in recent years. The problem of the control of insects injurious to plant life, on the other hand, has always been one of great difficulty, and in the past its solution has been considered mainly from a chemical standpoint. In recent years, however, biological methods have been extensively employed, but with varying success, by means of animal auxiliaries such as *Novius cardinalis*, a ladybird beetle parasitic on the scale insect—*Icerya purchasi*, and by the employment of entomophytic higher fungi and bacteria. We are concerned here only with the latter. The results obtained up till now have been of the most contradictory nature and the partial successes which have been recorded do not justify the belief that such methods promise to have widespread application. Indeed, failure has been so often the rule that the method has been discredited in many quarters where it was once warmly advocated.

Virulence.

The difficulty of raising the virulence of entomophytic bacteria so as to render them capable of infecting *per os* is very great; indeed, only in a few instances have successful experiments of this kind been reported. Virulence of an organism for insects does not rise progressively by passage through individuals of the same species, as it may do in the case of infections among the higher vertebrates. Nor is this difficult to understand when we consider the profound differences between vertebrates and insects. The infecting bacteria are, moreover, often normal symbionts in the blood of the insects concerned, and it has been shown by various workers (Paillot, 1922; Hollande and Moreau, 1922; and White, 1923) that different bacteria are connected with different species of insects, each having its own species of *Coccobacillus*. These coccobacilli are normal to the insect and perform some useful function for it; for example, they may help to liquefy the connective tissues in the period of moulting and to accumulate urates in the fat-body and Malphigian tubes. But under unfavourable conditions they change their behaviour and become parasites in the body of their host.

Pospolov (1926) has observed the development of bacterial septicæmia in locusts under experimental conditions of low temperature (20° C.) and humidity near to saturation. Probably the periods of moulting are exceptionally critical for insects in the early stages of their development, as at these times they succumb very easily to bacterial disease (septicæmia).

Symbiotic micro-organisms may multiply in the fat cells and accumulate the reserve products and urates. After the period of the diapause they lose their vitality and, becoming dissolved, are used as food reserves for the developing sexual glands and other imaginal tissues of the insect.

Survival of Bacteria during Metamorphosis.

Bacot (1914) has shown that the alimentary canal of the flea larva may become infected with bacteria, if they are mixed with its food, and that the infection so induced may persist until the resting period of the larva in the cocoon, but that there is no satisfactory evidence that such infection can survive the pupal stage. On the other hand, in the case of the house fly (*Musca domestica*), bacterial infection of the alimentary canal at the larval period has been shown to survive to the adult stage by Bacot (1911), Ledingham (1911) and Graham-Smith (1911), in the case of *Calliphora erythrocephala* by Graham-Smith (1911) and in a species of *Sarcophaga* by Nicholls (1912).

Mode of Infection.

The normal form of infection among insects is a septicæmia characterized by the invasion of the blood and tissues by the microbe which determines the infection, death supervening when the bacteria increase rapidly in the tissues.

Immunity.

Natural antimicrobial immunity among insects is of a dual nature and depends upon a complex of humoral and cellular origin (Paillot, 1922). The bactericidal action of the blood plasma, however, is by far the more important factor, the action of the blood cells (purely a passive one) not as a rule coming into play until the invading micro-organisms have first been transformed into granules by bactericidal action.

Immunization of insects against infection can only take place in cases where the bacteria against which it is desired to immunize such insects are sensitive to the bactericidal influences of the blood in the course of the first inoculation (granule formation and bacteriolysis). The methods employed are analogous to those used among the higher animals, but the immunity, which is more rapidly acquired than among the latter, is of a more evanescent character. The resulting immunization is specific for the organism.

Metalnikov (1920) has carried out a series of experiments with the larvæ of the bee moth (*Galleria mellonella*) to determine the immunity of insects against various classes of micro-organisms, and finds that the larvæ are absolutely immune to infection with *B. tuberculosis*, *B. diphtheriæ*, and *B. tetani*. On the other hand they do not offer the least resistance to, and die from even the smallest doses of *B. coli*, *B. pyocyaneus*, *B. prodigiosus*, *B. subtilis* and *B. proteus*.

Metalnikov and Gaschen (1921) have produced immunity against infection with *B. coli* and *B. proteus* by injection of vaccines and have shown that weak doses act more rapidly than strong ones. Furthermore, the resulting immunity is carried over to the adult stage.

Bacterial Diseases of Insects of Economic Value.

BACTERIAL PARASITES OF THE HONEY-BEE, *APIS MELLIFICA*.

In addition to other conditions, such as virus disease (see Vol. VII), affecting the brood (Sacbrood), and acaridian disease of the adult insect (Isle of Wight Disease), bees suffer from at least three well-defined diseases of bacterial origin: one affecting the adult stage (Bee Paratyphoid), and two the larval and pupal stages (American and European Foulbrood).

Bee Paratyphoid—B. paratyphosus alvei *Bahr.*

History and symptoms. The causal organism of bee paratyphoid was isolated by Bahr (1919) from the excreta of honey bees during the investigation of a severe outbreak of abdominal disease among these insects in the neighbourhood of Copenhagen. A similar infection has also been reported from Germany by Raebiger and Wiegert (1921). The principal symptoms observed are weakness, paralysis and sometimes diarrhœa, the bees dying in from twenty-four hours to several days after the first onset.

Causal organism. *B. paratyphosus alvei* is found in great numbers in the fæces of the sick bees, often in almost pure culture. The bacterium is a motile, oval, Gram-negative, non-sporing aerobe. It produces acid and gas in glucose, maltose, mannitol, arabinose and xylose peptone water, no change, however, being observed in lactose, saccharose, dulcitol and adonitol. Milk is unaltered. Brown moist colonies are formed on potato. Slight indole production is also observed. The organism is agglutinated to high titre by the homologous serum, but no agglutination takes place with *paratyphosus* A., *paratyphosus* B., *enteritidis* Gaertner, or *voldagsen* serum.

Mode of infection. The infection is probably carried from hive to hive by means of sick drones from other colonies, through the sale and purchase of bee-stocks containing ' carriers ', and through infected frames, utensils and beehives. The infection can easily be transferred to healthy bees by feeding with small portions of culture in 5 per cent. sugar solution. Wasps are apparently also susceptible, but mice, guinea-pigs and rats are immune.

Control. It is suggested that the uncontrolled purchase and sale of bees should, if possible, be restricted and that bee-stock breeding centres be established and kept under bacteriological supervision, so that bee-keepers may be assured of a supply of healthy queens and bee colonies.

American Foulbrood—B. larvæ *White.*

History and symptoms. American foulbrood or ropy foulbrood is an infectious disease of the brood of bees caused by *B. larvæ* (White, 1920[1]), the disease being characterized by the decided ropiness of the decayed brood and by a peculiar foul odour. The condition is widely spread, occurring in the United States, Canada, New Zealand and in various European countries, and is of much economic importance.

Infection first manifests itself about the time when the larvæ fill the cell and have begun pupation, and, as a rule, after the cells have been capped. After the death of the larvæ the cells are usually uncapped by the adult bees, the irregular condition of the brood comb, containing dead brood in such uncapped cells, being characteristic. The affected larvæ gradually turn brown and, as the process of decay continues, the mass becomes viscid. Later on the dead larvæ slowly lose their ropiness and become brown scales adherent to the base and lower sides of the cells, the caps of which, if not removed by the bees, are often sunken and perforated.

Causal organism. *B. larvæ* is a slender Gram-positive rod with slightly rounded ends and with a tendency to grow in chains. It will not grow on ordinary laboratory media, but may be propagated under aerobic conditions on media containing a sterile larval filtrate, or on egg-yolk agar.

The organism measures from $2{\cdot}5\mu$ to 5μ in length and about $0{\cdot}5\mu$ in breadth. It is motile, especially in young cultures, and possesses numerous peritrichous flagella. Spores are formed in three days on solid media. They are very resistant to most destructive agents, and may remain alive and virulent for years in the remains (scales) of the larvæ and pupæ dead of the disease, and in cultures that have become and remain dry. Carbohydrate media are not suitable for the growth of the organism, but when growth occurs, no visible gas is formed, though slight acid formation is observed in some media.

Mode of infection. The virus is transmitted primarily through the food of bees and possibly also through the water supply. Robbing from diseased colonies of the apiary or from neighbouring apiaries is the most likely method of natural transmission. It has been found by Vincens (1924) that the larvæ of the bee moth—*Galleria mellonella*—feed, without any ill-effect, on the dried-up bodies of infected larvæ, and enormous numbers of living spores of *B. larvæ* have been demonstrated in the excreta of these parasites. The larvæ of the bee moth, which travel from hive to hive, thus may also be a factor in the dissemination of the disease.

Infection takes place during the feeding-time period of the larvæ by means of the alimentary tract (mid-intestine), the brood dying in the larval, prepupal or early pupal stages. Adult bees, rabbits, guinea-pigs, rats, and evidently man also, are not susceptible to infection.

Diagnosis. American foulbrood can be readily differentiated from European foulbrood, as in the latter disease the brood dies in uncapped cells and the characteristic foul odour is absent. In Sacbrood the larvæ alone are attacked, the cells are always capped, the remains are sac-like, and viscosity is not present.

Control. This consists in the removal of all material capable of transmitting infection and thus eliminating the cause of the disease. The swarm should be transferred from the infected hive, and badly infected combs and brood should be destroyed.

European Foulbrood—B. pluton *White.*

History and symptoms. European foulbrood is an infectious disease of the brood of bees caused by *B. pluton*. It is characterized by the death of the brood before the time of capping, the yellow hue of recently dead larvæ and the brown shade of those longer dead, the irregularity of the brood and the absence of any disagreeable odour. The disease is fairly widely distributed in the United States and Canada, and also in Denmark, England, Germany, France and Switzerland. Death of infected larvæ may take place at any time from the fourth day of larval life to pupation. The brood nest presents an irregular appearance, capped cells and uncapped ones being found scattered over the brood frames. The dead larvæ lose their characteristic pearly whiteness and assume a yellowish colour, later becoming brownish. The decayed remains are not characteristically ropy as in American foulbrood.

Causal organism. The exciting cause of the disease appears to be *B. pluton* (White, 1920^2). The organism has, however, never been cultivated in artificial media. The morphology is somewhat variable. In early infections the bacilli appear as short rods in pairs or in chains, in later stages the predominating form is that of a lancet-shaped coccus of about 1μ in length, which form is present almost exclusively in the terminal stage of the disease. The organism colours uniformly with the aniline dyes and is Gram-positive. It does not produce spores and is a facultative anaerobe.

Mode of infection. *B. pluton* gains entrance to the larva by way of the buccal cavity, the growth and multiplication of the parasite taking place within the mid-intestine, the general tissues of the larva not being invaded. The incubation period is about three days. The adult bee is never affected, and rabbits, guinea-pigs, pigeons and white rats appear to be refractory to inoculation with suspensions of larvæ dead of European foulbrood; nor, so far as is known, are other insects susceptible. Man also is evidently immune to infection.

Secondary invaders, chiefly *B. alvei*, *B. eurydice*, *Streptococcus apis*, and occasionally *B. orpheus*, are encountered at various stages of the disease and during the decay of the larvæ. They appear to have no pathological significance, rarely, if ever, invading the tissues until the larvæ are dead or nearly so.

Infection is most probably transmitted in nature by robbing from diseased colonies of the apiary or from neighbouring apiaries.

Control. Methods of control are essentially the same as for American foulbrood.

Miscellaneous Infections of Bees.

Infectious diarrhœa. Two organisms, *B. coli apium* and *Proteus alveicola*, are described by Serbinov (1915) as being the cause of infectious diarrhœa of bees during the spring of the year, and were observed for several years on the Gulf of Finland. The organisms are described as being infective to mice by peritoneal inoculation.

BACTERIAL DISEASES AFFECTING THE SILKWORM—*SERICARIA (BOMBYX) MORI.*

The silk industry has suffered repeatedly from epizootic infections, which have at times threatened its entire prosperity. In addition to protozoal disease ('pébrine' caused by the microsporidian, *Nosema bombycis*) and filter-passing infections (Jaundice of the Silkworm, see Vol. VII), silkworms suffer from numerous bacterial infections. These are generally described under the name 'Flacherie', which, according to Paillot (1926^1) is due to a number of infections of the intestinal tract.

Flacherie of the Silkworm—Streptococcus bombycis *Sartirana and Paccanaro.*

History and symptoms. This disease, one of the scourges of sericulture, was first recognized by Pasteur. The diseased larvæ refuse to eat and become torpid. They do not spin cocoons but stretch themselves out and remain motionless till death supervenes. Post-mortem changes soon take place; the worms assume a blackish colour and within forty-eight hours their bodies are filled with a brownish fluid swarming with bacteria. No 'polyhedral' bodies are present and thus the condition is readily distinguished from 'pébrine' and from 'grasserie'.

Causal organism. The causal organism—*Streptococcus bombycis* (Sartirana and Paccanaro, 1906) forms chains of small Gram-positive cocci some 0·9μ in diameter, the chains being from 5μ to 12μ long. Colonies upon agar are deep brown in colour, small and round, with a slightly undulating contour. Gelatin is not liquefied and milk is not curdled. Indole production is negative. The streptococcus is a facultative anaerobe, and the optimum temperature for growth appears to be about 37° C., but development also takes place at 20° C. The cultures show great vitality.

It is stated by Paillot (1926[1]) that symptoms of flacherie may be produced by other organisms parasitic to the silkworm. He observed flacherie symptoms in silkworms infected with a Gram-positive sporulating bacillus, which caused death in 4 or 5 hours after inoculation at 22° C. The larvæ blackened rapidly after death. The bacillus proved incapable of inducing infection *per os*. He also described a Gram-negative sporing bacillus which was found to be incapable of growth on ordinary laboratory media. This organism was often found in association with *Streptococcus bombycis*, which latter he confirms as the principal factor in the production of flacherie infection.

Mode of infection. Infection takes place through feeding on mulberry leaves infected either through fæcal or dust contamination. When the infected larvæ survive and develop into moths and lay eggs, the arising brood is always infected. Factors favouring the development and spread of infection are overcrowding, lack of fresh air and high humidity. The condition is easily recognized, the characteristic micro-organism being always present in tissues from the digestive cavity.

Control. This consists principally in selecting only healthy strains of moths for breeding purposes and only using moths for seeding that come from strains that have been examined bacteriologically.

Bacterial Disease of the Silkworm—Glaser.

History. A bacterial disease of the silkworm having the same external appearances as 'Flacherie', and originating in the alimentary tract, has been described by Glaser (1924). It has no relation to the polyhedral disease known as 'jaundice', 'gelbsucht' or 'grasserie', nor with the microsporidian infection—'pébrine'.

Causal organism. This is described as a Gram-negative, motile bacillus forming capsules but not spores. It produces a white, abundant, glistening growth on agar, reduces and peptonizes milk and liquefies gelatin. Indole is not produced, but a positive carbinol reaction is given. Acid and gas are formed in glucose, saccharose, maltose and mannitol peptone water, but no change is observed in lactose and dulcitol.

Mode of infection. Infection is not conveyed through the egg, as is stated to occur in true flacherie of streptococcal origin, and is presumably spread by the fæces of dead or dying individuals.

Miscellaneous Infections of the Silkworm.

Coccobacillosis—B. bombycis *Chatton.* This organism—isolated by Chatton (1913) from epizootic disease among silkworms, resembles morphologically *B. melolonthæ* Chatton (see p. 126), but is devoid of fluorescence on agar. It kills silkworms in from 12 to 24 hours after injection into the body cavity. About 15 per cent. of experimental infections *per os* are successful. As with other members of this group (coccobacilli), the organism appears to be a normal inhabitant of the alimentary tract of the host.

The disease is certainly distinct from 'grasserie', no particular external symptoms are noticeable before death, and polyhedral cells are not observed. Neither does it correspond to 'flacherie', but it appears to be a distinct pathological entity.

'La Gattine'. Paillot (1926[2]) also incriminates *Streptococcus bombycis* as the ætiological factor in the production of another morbid condition among silkworms, known in France as 'La Gattine', and in Italy as 'Macilenza', characterized generally by the swelling of the anterior body wall, which becomes more or less translucent. An organism agreeing with *Streptococcus bombycis* was isolated from the intestinal contents.

Bacterial Diseases of Injurious Insects.

BACTERIAL PARASITES OF THE LEPIDOPTERA.

Japanese 'Gipsy Moth' Disease—Streptococcus disparis *Glaser.*

History and symptoms. An infectious disease of the caterpillars of the Japanese race of the gipsy moth—*Porthetria* (*Lymantria*) *dispar*, which spread to cultures of the American race, has been described by Glaser (1918[1]). The disease is entirely distinct from wilt (polyhedral disease), which is caused by a filterable virus (see Vol. VII), and also from 'pébrine', a microsporidian disease caused by *Plistophora schubergi* (Zwölfer, 1927), the causative agent being *Streptococcus disparis*.

The symptoms among infected caterpillars consist of diarrhœa, loss of appetite and muscular co-ordination. After death the insect hangs in a flaccid condition by its prolegs, with the appearance of death from wilt. The skin, however, does not rupture, as in wilt. The bacterium enters the alimentary tract of the caterpillar along with the ingested food. During the latter stages of the disease and after death, it invades practically all the tissues.

Causal organism. Streptococcus disparis is a capsulated, non-motile, Gram-positive aerobic organism of diameter less than 1μ. Chains of from three to four units are frequent in liquid media. The cultural characters are as follows: Scanty growth on agar in five days at 35° C., but abundant, glistening growth on potato agar under the same conditions. Gelatin is not liquefied. Indole is not produced. Litmus milk is made acid and coagulated later, but there is no peptonization of the clot. Acid is formed but no gas in glucose, lactose, saccharose, maltose, mannitol, adonitol and dulcitol. Facultative anaerobe.

Mode of infection. Healthy caterpillars become infected by eating food soiled by the fæces of infected larvæ. Field experiments have been conducted with *Streptococcus disparis* in sections of the gipsy-moth-infected territory in the United States with success, severe epizootics being created on two occasions. The streptococcus is not pathogenic to silkworms (*Sericaria mori*) nor to 'army worms' (*Cirphis unipuncta*) nor to human beings, guinea-pigs or rabbits. It is pathogenic to the American, European and Japanese races of the gipsy moth.

Pseudograsserie of the Gipsy Moth Caterpillar—B. lymantricola adiposus *Paillot.*

History and symptoms. Two separate organisms were isolated by Paillot (1919[1]) from a caterpillar of *Porthetria* (*Lymantria*) *dispar* which presented the external symptoms both of 'grasserie' and 'flacherie' and were designated by him as *B. lymantricola adiposus* and *B. lymantriæ* respectively, the former alone being responsible for the condition described. A few hours after experimental inoculation with a pure culture of *B. lymantricola adiposus* the blood presents the same milky appearance as with larvæ attacked with 'grasserie', but the effect is produced by fat globules in the blood and not by polyhedral cells, as in true 'grasserie'. The pathogenic action of the bacillus thus manifests itself principally in the disorganization of the adipose tissue.

Causal organism. The organism is a coccobacillus 1μ in diameter and 2μ long, motile, Gram-negative and showing marked pleomorphism in various media. It forms large, round, yellowish-white colonies on agar. Milk is coagulated, inspissated serum is digested, and gelatin rapidly liquefied. Glucose, lactose, saccharose, mannitol, maltose, galactose, dulcitol and arabinose are fermented.

Pathogenicity. The bacillus is pathogenic for all species of insects, including the caterpillars of *Vanessa urticæ*, the brown-tailed moth (*Nygmia phæorrhœa*) and the silkworm (*Sericaria mori*).

Miscellaneous Infections of the 'Gipsy Moth'.

Coccobacillosis. Coccobacilli, causing a fatal septicæmia among caterpillars of the gipsy moth, have been described by Picard and Blanc (1913[1]) in the south of France. Similar organisms were afterwards isolated and described by Paillot (1922) as *B. lymantriæ* α Picard and Blanc. This author has also described two other organisms—*Diplococcus liparis* β and *B. liparis*, in association with the foregoing. They appear to have but little pathogenic importance.

Bacillary Septicæmia of Caterpillars of Arctia caja, Coccobacillus cajæ *Picard and Blanc.*

History and symptoms. A heavy infestation of the vineyards of the south of France occasioned by the 'Woolly Bear' caterpillar (*Arctia caja*) was almost completely wiped out through the action of a fungus—*Empusa aulicæ*—and by a septicæmia of bacillary origin. The dead caterpillars became flaccid and emitted a nauseating odour, the digestive tube being empty of its contents and containing only a clear liquid, often free from micro-organisms. A coccobacillus was obtained in pure culture from the blood and with it the disease was experimentally reproduced (Picard and Blanc, 1913[2]).

Causal organism. *Coccobacillus cajæ* measures about 1·5μ in length, is motile, Gram-negative and shows bi-polar straining. Optimum growth takes place at 25°C. The cultures exhale an odour of H_2S, take on a greenish fluorescence, but do not contain pyocyanin. Gelatin is rapidly liquefied.

The organism appears to belong to the same group as *C. acridiorum* d'Herelle. It is, however, a parasite of the blood of the caterpillar and not of the digestive tube. Caterpillars, having received by ingestion some drops of culture by means of a pipette introduced into the pharynx, die after a few hours with their blood invaded by the bacilli.

Pathogenicity. Caterpillars of *Porthesia chrysorrhea* are readily infected with the organism. Certain aquatic coleoptera and hemiptera appear to be immune. The tree frog (*Hyla arborea*) is, however, rapidly destroyed with an intense bacillary septicæmia.

Hornworm Septicæmia—B. sphingidis *White.*

History and symptoms. The larvæ of the sphingids, *Protoparce sexta* and *P. quinquemaculata* (hornworms of tobacco or tomato) are occasionally attacked by a septicæmia which sometimes proves fatal. The disease, when inoculated by puncture, runs a course of from 18 hours to 2 or 3 days. The principal symptoms are loss of appetite, stupor, diarrhœa and thin vomitus. After death the larvæ become black and shrivelled.

Causal organism. This is described by White (1923[1]) as *B. sphingidis*. The organism, a Gram-negative, non-sporing rod of up to 1·5μ in length, produces acid in glucose, mannitol, saccharose, maltose, arabinose, xylose, dextrin and salicin. A trace of acid is given in lactose peptone water but there is no action on dulcitol. Traces of gas are observed in glucose and saccharose. It is actively motile and rapidly liquefies gelatin. A homologous serum agglutinates the organism up to a titre of 1 in 4,000, but has no action on the closely related *B. noctuarum* (see p. 122) or *Coccobacillus acridiorum* (see p. 123). *B. sphingidis* is readily destroyed by heat, drying, direct sunlight and chemical action, but lives for a long time in a moist environment at room temperature. No appreciable loss of virulence has been noticed in cultures of the bacillus kept for years on artificial media.

Mode of infection. Little is known of the transmission of the disease in nature but it probably takes place by means of the alimentary tract : the point of entry is not definitely known, but the bacillus is frequently found between the folds of the stomach wall. Infection seems most likely to occur during the fifth larval instar, a high temperature being in all probability a predisposing cause.

A comparatively small proportion of healthy larvæ die following infection with the virus by feeding, and probably few die of the disease in nature during the active growing season of their food plants, but practically all of them succumb following puncture inoculation.

Pathogenicity. Cutworms, the larva of the catalpa moth (*Ceratomia catalpæ*) and of the silkworm moth (*Sericaria mori*) and grasshoppers are all very susceptible to puncture inoculation and die rapidly from septicæmia. No insect so far tested has proved to be immune. There is need for a much more comprehensive study of this group of insect diseases.

Cutworm Septicæmia—B. noctuarum *White.*

History and symptoms. White (1923[2]) has also made a study of a septicæmia of cutworms (larvæ of *Peridromia* and *Agrotis,* parasites of young cotton) and has shown that the disease is due to an organism which he describes as *B. noctuarum.* The condition, when produced by inoculation puncture, lasts from two to four days and ends fatally.

Causal organism. The organism, a Gram-negative, motile, non-sporing, aerobic bacillus, closely resembles *B. sphingidis* (see p. 121) in its cultural characters, but may be differentiated from it and from *Coccobacillus acridiorum* by serological tests. A homologous serum agglutinates the organisms up to a titre of 1 in 3,200, but gives a negative result with the other organisms mentioned.

Pathogenicity. The pathogenicity of *B. noctuarum* for other insects is essentially the same as that of *B. sphingidis.*

Miscellaneous Infections of Caterpillars.

Bacterial infections of caterpillars of Pieris brassicæ. No less than nine different species of bacteria infecting the caterpillars of the ' cabbage white ' butterfly were observed by Paillot (1919[2]) during a formidable invasion of the cabbage-growing district of the south of France with these pests. They are described under the names *B. pieris fluorescens, B. pieris liquefaciens* α and β, *B. pieris non-liquefaciens* α and β, *B. pieris agilis, Diplococcus pieris, Diplobacillus pieris* and *B. proteidis.* It is considered that bacterial parasites play only a secondary role in the destruction of the larvæ, which are principally killed off by the parasitic insect *Apanteles glomeratus.* In years when the *Apanteles* are rare, the bacterial infections are also rarely encountered. These parasites, therefore, appear to predispose the caterpillars to bacterial infection.

Bacterial infection of larvæ of Gortyna ochracea—B. gortynæ *Paillot.* Paillot (1913) has succeeded in isolating a bacillus—*B. gortynae*—from the caterpillars of *Gortyna ochracea* (a pest of the artichoke) which causes an epizootic among these insects. At first the diseased caterpillars do not show symptoms, but later they lose the use of all their legs, except the first pair, and the posterior part of the body seems to lose sensation. After death the body quickly decomposes. On microscopical examination many motile coccobacilli are seen in the blood. The bacilli, when present in the tissues of healthy caterpillars, seem to render them immune.

In the same communication Paillot also describes two bacterial parasites of the caterpillar of the ' painted lady ' butterfly—*Pyrameis* (*Vanessa*) *cardui,* parasitic on the leaves of the artichoke—under the names *B. pyrameis I* and *II.*

Bacterial disease of the larvæ of the Bee Moth, Galleria mellonella. Kitajima and Metalnikov (1923) report a fatal epizootic among the larvæ of the bee moth, *Galleria mellonella,* in the laboratory. Two organisms were isolated, a micrococcus and *B. galleriæ.* Both were found to be lethal to the insects.

Bacterial parasites of the Processionary Moth Caterpillar, Cnethocampa pityocampa. Dufrenoy (1919) has described three organisms, a Gram-negative bacterium—*B. pityocampæ,* and two streptococci—*Streptococcus pityocampæ* α and β attacking the processionary moth caterpillar. The caterpillars die within two to four days after inoculation with *Streptococcus pityocampae* α, the muscles being infiltrated with the coccus, the fibres degenerating and losing their striation.

Septicæmia of caterpillars of Malacosoma castrensis—Coccobacillus insectorum *var.* malacosoma. Pathogenic bacilli have been found in the blood of the caterpillars of *Malacosoma castrensis* and have been described by Hollande and Vernier (1920). The organisms are motile, Gram-negative rods, 3μ to 5μ in length, and exhibit polar staining with methylene blue. Indole is not produced, and milk is curdled. Blood serum is digested with a putrid odour. After death the bodies of the caterpillars become swollen and exhale a strongly ammoniacal smell. It is suggested that the organism is a mutant of *B. fluorescens liquefaciens.*

BACTERIAL PARASITES OF THE ORTHOPTERA.

Bacterial Disease of Locusts—Coccobacillus acridiorum *D'Herelle.*

History and symptoms. This organism was first isolated by d'Herelle (1911) in Yucatan, Mexico, from the excreta of locusts of the species *Schistocerca americana* (*pallens*), the infecting organism being discovered in the intestinal contents of dead locusts, but never found in healthy specimens. In this year the epizootic was confined to definite bands of locusts, but in the next year all the swarms appeared to be attacked and subsequently the locust invasion ceased.

In natural infections the incubation period varies from 1 to 48 hours, according to the virulence of the infecting strain, the individual resistance of the locust, and the climatic conditions. Indeed, in the opinion of Mereshkovsky (1925), Pospolov (1926) and others, *Coccobacillus acridiorum* is a normal symbiont in the blood of locusts, which can, only under favourable conditions of temperature and humidity, become a dangerous parasite.

At first the contents of the chyle stomach become liquid and assume a dark colour resembling coagulated blood. The intestinal contents also liquefy, turn yellow and then blackish; light pressure on the abdomen at this stage causes the extrusion of liquefied intestinal contents. Several hours later the locust falls on its side, the legs move spasmodically and death supervenes after a comatose period lasting from minutes to several hours. All the tissues of the locust are invaded by the organism and the body putrefies rapidly after death.

Causal organism. Coccobacillus acridiorum, the causal organism of the Mexican locust epizootic, is a pleomorphic bacillus, coccoid forms about 0·6μ long and bacillary forms of from 0·9μ to 1·5μ occurring in the same culture. The organism is very motile, showing numerous peritrichous flagella. It exhibits marked bi-polar staining, is aerobic and does not form spores. In young cultures and in the intestinal contents of the locust the coccoid forms are met with almost exclusively. It develops at from 16° C. to 43° C., and gives a rapid growth in broth at 37° C. After several hours a pellicle appears on the surface of the broth cultures, which clears after some weeks, leaving a copious deposit. On agar the young colonies are of waxy appearance and are of 2 to 3 mm. in diameter after 18 hours. Some strains liquefy gelatin, others do not. Milk is coagulated and rendered strongly alkaline. The organism ferments lactose, glucose, mannitol and saccharose with production of gas, but has no action on dulcitol. Indole is not formed.

Mode of infection. Infection takes place through the alimentary canal. Natural methods of spread of infection consist in the contamination of herbage with dejecta from diseased insects, cannibalism and the soiling of the eggs of the new brood with infected excretions. A certain number of locusts in the swarm perhaps act as healthy ' carriers '.

Pathogenicity. The natural epizootic in Yucatan was among locusts of the genus *Schistocerca*, and the bacteria appear to be more effective against locusts belonging to this genus than against other genera of the Locustidæ. Species of other genera experimentally proved to be susceptible to infection include *Caloptenus* in Argentina and *Stauronotus maroccanus* in Algeria. The Philippine locust *Pachytylus* (*Locusta*) *migratoroides* has, according to Mackie (1913), given negative results with *C. acridiorum* and experiments conducted with *Zonocerus elegans*, a non-migrating South African species, have not been encouraging (Lounsbury, 1913). Sergent and Lhéritier (1914) found that the virus can be increased to kill *Stauronotus maroccanus* in four hours, that groups of these insects may be exterminated by spraying the nocturnal resting-places with cultures of *C. acridiorum*, but that the induced infections show only a slight tendency to spread to other groups. Good results have been obtained in French Guinea by Cheyssial (1922) against *Stauronotus* and *Caloptenus*, while, on the other hand, Kraus (1921) asserts that *C. acridiorum* being, as he states, a normal inhabitant of the intestinal tract of locusts, is valueless as a means of locust destruction.

Several species of ants have also been found to be susceptible, and experiments have been successfully carried out against caterpillars infesting the cotton-fields in Argentina. Fowls, guinea-pigs, rabbits, cows, sheep and man are refractory to infection, but the sewer rat is susceptible to subcutaneous inoculation, dying in three days and a half with generalized septicæmia.

The organism rapidly loses its virulence in culture, but attenuated cultures can be exalted in virulence by the injection and passage through the abdominal cavities of locusts. After three passages the strain commences to kill again and after five or six passages will kill *per os* in 8 to 12 hours. d'Herelle (1915) finds that when the virulence of the coccobacillus has been exalted by passage so as to kill in 8 hours, if the bodies are carefully dried over sulphuric acid *in vitro* and sealed off in glass tubes, the virus will remain potent for two years.

Locusts which have survived inoculation with an attenuated culture are rendered immune to virulent strains. In nature, 20 to 25 per cent. of individuals appear to be naturally immune.

Miscellaneous Infections of Locusts.

B. poncei, *Glaser.* A systematic study of the organisms distributed under the name *Coccobacillus acridiorum* has been undertaken by Glaser (1918^2), who has described a new member of this group, *B. poncei* from Honduras, pathogenic to *Melanopus femur-rubrum* and *Encoptolophus sordidus.*

Micrococcus acridicida, *Kuffernath.* An organism, *Micrococcus acridicida,* causing spontaneous disease in locusts in Greece, and also pathogenic for the larvæ of *Pieris rapæ* and *Locusta viridissima,* has been reported by Kuffernath (1921). It is closely allied to *Staphylococcus pyogenes.* Inoculated locusts and others fed on vine leaves which had been sprayed with an emulsion of the bacteria, die within 48 hours from septicæmia.

BACTERIAL PARASITES OF THE COLEOPTERA.

Bacterial Disease of June Beetle Larvæ, Lachnosterna *Spp.*—Micrococcus nigrofaciens *Northrup.*

History and symptoms. This disease has been found by Northrup (1914) to be common everywhere in the United States where 'white grubs' of this and related species are found. The disease also occurs in Porto Rico.

The normal larvæ are white, firm, and covered with conspicuous hairs, while badly diseased grubs are almost entirely black and appear to be in a state of putrefaction, even though they still show signs of life. In the earlier stages the diseased larvæ have black shiny spots located along the joints of the legs, on the spiracles, and upon the dorsal and ventral surfaces of the body. In some cases the infection commences at the tip of the leg, and as it passes up, the limbs blacken and drop off segment by segment, leaving shiny, black and sometimes swollen stumps.

Causal organism. The causal organism, *M. nigrofaciens,* is a pleomorphic, Gram-positive coccus of varying size. It grows abundantly upon agar, forming flat, glistening, opaque, pale orange colonies. Gelatin is liquefied and litmus milk acidified. Lactose, glucose and saccharose are not fermented. A moderate amount of indole is produced in peptone broth.

Mode of infection. The organism is probably common in soil, and infection takes place through abrasions. Under natural conditions the larvæ bite one another, especially if they are very numerous in any one place; this may account for the rapid spread of the disease. Excessive humidity of the soil also favours the progress of infection. *M. nigrofaciens*, on account of limiting environmental conditions, does not readily lend itself as a means of control of white grubs.

Pathogenicity. The larvæ of the southern June beetle (*Allorhina nitida*) is susceptible to this infection, but less so than the white grub. The American cockroach (*Periplaneta americana*) is also slightly susceptible. The virus is but doubtfully pathogenic to ' angle worms ', and guinea-pigs and rabbits inoculated subcutaneously and intraperitoneally suffer no ill-effects.

Miscellaneous Infections of Coleoptera.

Coccobacillary septicæmia of the cockchafer, Melolontha melolontha. *Coccobacillus melolonthæ*, described by Chatton (1913), is related to *Coccobacillus acridiorum* (see p. 123), but is distinguished by its fluorescence upon agar after five or six days. It is also distinguished by its pathogenic action on the silkworm. Injected into the general body cavity it kills the cockchafer in from 12 to 36 hours, but is incapable of conveying infection *per os*.

The organism is also found in the digestive tract in 75 per cent. of healthy cockchafers: it only passes into the general body cavity and produces the characteristic symptoms under special and unknown conditions. On account of its pathogenicity for the silkworm it might be considered unwise to employ the bacillus experimentally as a destructive agent in a silk-producing district.

Other organisms producing cockchafer septicæmia. Paillot (1922) has carried out a series of investigations concerning the bacterial parasites of the cockchafer, and it appears that this insect may be parasitized by a number of bacterial species, differing often with the locality infested. In general, the sick insects suffer from a septicæmia, which may be recognized by the non-coagulability of the blood, which becomes in addition more or less turbid. Paillot describes no less than eleven species of such organisms—*B. melolonthæ liquefaciens* α, β and γ, *B. melolonthæ non-liquefaciens* α, β, γ, δ and ε, all of which are Gram-negative; a Gram-positive, sporing, motile bacillus resembling *B. anthracis* (*B. hoplosternus*); a Gram-positive non-motile bacillus (*Diplobacillus melolonthæ*); and a Gram-positive coccus (*Diplococcus melolonthæ*). All these organisms, particularly the gelatin liquefiers, are pathogenic to the cockchafer and to various other insects.

Bacterial parasite of the beetroot weevil, Conorrhynchus mendicus—B. cleoni. *B. cleoni* is stated by Picard (1914) to be a natural enemy of the beetroot pest, *Conorrhyncus mendicus*, a large weevil appearing in the spring and devouring the young leaves. The bacillus is said to resemble *B. coli*, but liquefies gelatin.

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CHAPTER VII. BACTERIAL DISEASES OF PLANTS.

BY S. G. PAINE
(IMPERIAL COLLEGE OF SCIENCE AND TECHNOLOGY, LONDON).

Introduction.

THE earliest suggestion that bacteria might cause disease in plants was that of Burrill in 1878, who ascribed a disease of pears, 'The Fire Blight Disease', to a bacterial origin; this was confirmed later by Waite (1898), who isolated the organism and proved its pathogenicity. The discovery of other diseases followed, for example: Black Rot of Cabbage by Pammel (1895) and Soft Rot of Vegetables by L. R. Jones (1901), but, in spite of these discoveries the Germ Theory of Disease was not readily accepted for plants, and there seems to have been a great deal of doubt as to the part which the bacteria played in the production of the diseases. This led to the controversy, now become almost classic, between Alfred Fischer (1897, 1899) and Erwin F. Smith (1899^1, 1899^2, 1901), in which the latter succeeded in demonstrating the importance of bacteria as producers of plant disease. At the present time many diseases of plants are known to be caused by specific organisms. In every case where a disease has been attributed to an organism the pathogenicity of the latter has been established by isolation, inoculation of healthy plants, re-isolation in pure culture and re-inoculation of the host, so that no reasonable doubt exists as to the ætiology in the various diseases.

Types of Bacterial Disease.

The following is an account of the main types of bacterial disease.

The Soft Rots. Among the most destructive of plant diseases are those in which the plant tissue is reduced to a soft, wet, pulpy condition usually recognized as a state of rottenness. This may not be due to a specific disease but may be a secondary phenomenon following the attack on the plant of some insect pest or parasitic fungus. The bacteria in this case are common soil organisms of saprophytic habit which find a suitable pabulum in the sap of cells exposed to them by the invading insect or fungus. Having obtained a foothold many of these soil organisms produce ammonia in such quantity as to kill the neighbouring plant cells; thus they rapidly progress through the tissue, advancing always in a substratum of cells whose death has been brought about by toxic volatile products liberated from the proteins of the plant by the action of the bacteria.

The soft rots proper are produced by bacterial inhabitants of the soil, which differ from the common run of soil organisms in having the power to attack healthy plant tissue by means of an exocellular enzyme which they excrete into the surrounding medium. It is capable of dissolving the pectins, of which the middle lamella substance, the cementing material of the plant tissue, is composed. The pectic substances are converted into soluble materials, probably sugars, which are utilized as food by the bacteria. The bacteria being motile rapidly insinuate themselves into the tissue, the structure being reduced thereby to a mass of separate cells suspended in a bacterial slime.

The enzyme is capable of precipitation by alcohol from a broth culture of the rot-producing organism ; and, as a powder, will retain its activity for more than twelve months if kept perfectly dry. A 5 per cent. aqueous solution of the dry powder can be shown to produce the breakdown of the tissue of the host plant in a manner exactly similar to that of the specific organism. The effect of the enzyme can be demonstrated also in the following way: A colony of the organism in Petri dish culture is allowed to assume a diameter of three or four millimetres, and the block of agar which bears it is carefully cut out and placed aseptically on the surface of a slice of living turnip tissue. On examination after incubating overnight the surface of the turnip lying directly beneath the colony, but separated from it by the layer of agar, will be found in a rotten condition, and, if the experiment has been properly carried out, the rotted tissue can be shown to be free from contamination with living organisms, the observed effect being brought about by the enzyme which has diffused through the agar layer that separated the tissue from the bacteria.

The chief organisms which bring about this type of disease in England are *Bacillus carotovorus*, the most common cause of rot among vegetables, *Bacillus atrosepticus*, which produces the so-called ' Blackleg ' disease of potato, and *Bacillus rhaponticum*, responsible for considerable loss to the rhubarb grower. All these are very similar in many respects and are evidently closely related. It would indeed be interesting to discover that they had a common ancestor among the soil organisms. It cannot be assumed that plant pathogens did so arise, but some support for the view is afforded by the finding of Berridge and Mehta (1924) that *Pseudomonas pyocyaneus*, under laboratory conditions, can readily be made to produce a rot of lettuce similar to that ascribed by Brown (1918) to *Bacterium marginale*, and that the latter is so similar to the former as to be almost, if not quite, indistinguishable from it. The forerunner of the rot-producers might possibly be found in *Bacillus coli.*

The organisms named are all bacilli of the non-sporing kind and coliform in shape and general character ; they differ from the colon bacillus in so many ways that it seems unlikely that the relationship is anything more than a superficial resemblance ; the differences are not so great, however, as to place their evolution from *B. coli* beyond the region of possibility (see table on page 130).

Vascular diseases. Diseases are common in which the sap current of the plant is interrupted by the accumulation of bacteria in the conducting

COMPARISON OF THE CHARACTERISTICS OF VARIOUS ROT-PRODUCING ORGANISMS WITH THOSE OF *BACILLUS COLI COMMUNIOR*.

	Bacillus atrosepticus	*Bacillus solanisaprus*	*Bacillus carotovorus*	*Bacillus rhaponticum*	*Bacillus coli communior*
Liquefaction of gelatin ..	+	doubtful, when first described liquefaction only occurred after 35 days.	+	−	−
Glucose broth	acid + gas −	acid + gas very slight	acid + gas +	acid + gas −	acid + gas +
Lactose broth	acid + gas −	acid + gas very slight	acid + gas +	acid + gas −	acid + gas +
Sucrose broth	acid + gas −	acid + gas very slight	acid + gas +	acid + gas −	acid + gas +
Reduction of nitrates ..	+	+	+	+	+
Diastatic action.	−	very slight	strong		strong
Casein digestion.	+	+	+		−
Indole production	−	−	−	−	+
Colony on MacConkey ..	white	white	white	white	red
Optimum temperature ..	30° C.	25–30° C.	25° C.	25° C.	37° C.
Growth at 37° C.	none	very slight	slight	none	good
Thermal death-point ..	47° C.	50° C.	50° C.		56° C.

B. atrosepticus, *B. solanisaprus* and *B. carotovorus* are closely related organisms. Jennison (1923) holds them to be identical, but cultural and serological differences shown by Lacey (1926) and agglutination phenomena shown by Berridge (1926), seem to warrant their continued separation as distinct species.

elements of the vascular system. The extent to which the flow of sap is restricted varies in different diseases; it may be complete, with the result that the plant rapidly wilts, or it may be partial, in which case the plant becomes sickly, makes poor headway, often exhibits one-sided growth, and finally succumbs, sometimes after many weeks. Death is frequently due to the attack upon it in its weakened condition of some other organism.

An example of the former is the Cucumber Wilt Disease caused by *Bacillus tracheiphilus*. This organism is heavily capsulated and forms a viscous zoogl
œa so ropy in its consistency that separate threads of it can be drawn from the vascular bundles when the finger is applied to the cut end of a diseased stem, and carried to a distance of four or five inches without breaking.

An example of the latter is Stewart's disease of maize caused by *Aplanobacter stewarti*. Infection usually takes place in the seedling stage. Some of the plants are destroyed at this stage, but many of them reach a height of several feet before showing signs of disease.

In many instances of vascular disease, as in the Brown Rot of potato and tobacco caused by *Pseudomonas solanacearum* (*Bacterium solanacearum* E.F.S.) and in the Brown Rot of cabbage and turnip due to *Pseudomonas campestris*, the organism having established itself in the vascular bundles, and probably having exhausted the supply of food there, makes excursions into the surrounding mesophyll, destroying the cells, and forming cavities which gradually extend until the greater part of the storage tissue becomes involved in a form of dry-rot.

Intumescence diseases. Certain species of bacteria exercise an influence upon the meristematic tissue of their host plants, causing them to develop abnormally, and to grow out in the form of galls or tumours. These galls may remain small, as in the root tubercles of the *Leguminoseæ* and the leaf galls of the *Rubiaceæ*, or they may assume very large proportions as in the Crown Gall Disease. In the latter case it is no unusual thing to find a root, sugar beet for instance, carrying a tumour equal in size to, or, in fact, even larger than itself. Such growths bear a certain resemblance to cancer in animals, and the late Erwin F. Smith spent much energy in his later years trying to prove that plant and animal cancers were similar in origin. His main argument was centred upon the production of secondary tumours arising in the affected plant at some distance from the primary tumour. These he believed to be the plant equivalent of metastasis in animal cancer, being caused by the transference of one or a few cells of the primary growth by way of a conducting element into the position where the secondary tumour makes its appearance.

Such, however, is shown by Robinson and Walkden (1913) and Riker (1923) not to be the case; these workers independently and simultaneously found that the so-called secondary tumours were really belated manifestations of the primary infection, that in these cases the original infection was at or near the growing point of the plant, and some bacteria were carried by the growing apex to a point remote from the point of infection.

It is interesting to find in these intumescence diseases that the presence of bacteria irritates the cells and causes rapid division. The bacteria need not be within the cells to produce this effect. In Crown Gall *Bacillus tumefaciens* is found on the outside of the gall and in spaces between the cells, the so-called tumour strands; it must be assumed that diffusion of chemical stimuli takes place, and this assumption is supported by the experiments of E. F. Smith (1917), in which he produced proliferation of the pith in the hollow stem of *Ricinus* by the introduction through the walls of capillary glass tubes filled with volatile substances such as ammonia, formic acid and acetic acids.

Local lesions. Included under this head are a number of bacterial diseases, which, as the name implies, are confined within certain narrow limits. In the simplest case, that of leaf-spot diseases, the attack is restricted to a small area around the point of entry. The cells involved become heavily infected and strongly discoloured; they soon dry up, and, breaking away from the healthy tissue around them, frequently fall out, producing a shot-hole effect. It is curious that the organisms so vigorous in their first attack should have so little power to penetrate further into the tissue; possible reasons for this behaviour are discussed later. In other cases the bacteria make extensive passages through the plant tissue, selecting a narrow path among the cells and leaving the neighbouring cells entirely unaffected; where such passages approach the exterior of the plant, disease shows itself as streaks and spots caused through discoloration and shrinkage of the cells. Such is Tomato Stripe Disease caused by *Bacillus lathyri.* In this case the organism is present already in the seed so that the young seedling starts life at a disadvantage. This frequently results in early death, but, the organism being a comparatively ineffective parasite, many plants grow to a considerable size, and, in fact, often reach the fruiting stage. In these cases it is interesting to trace the path taken by the bacteria. At first they pass up the stem in the region of the pith, and near to, but not within, the protoxylem elements. From here they proceed by the medullary rays and gain access to the cortex. Having reached the cortex they take the shortest path to the exterior, where they select a narrow belt of cells lying just beneath the epidermis; streaks formed in this way may be anything from one-half to four or five inches in length and about one-eighth of an inch in breadth. In the leaf the organism enters the mesophyll from the vein system, singles out certain patches of tissue and produces thus a blotchy appearance on the leaves. Similarly, the appearance of the fruit is marred by brown sunken discs, rings or sometimes horseshoe markings. Reasons for this kind of selective action are hard to conceive and at present are entirely unknown.

Mode of Entry of Bacteria into the Plant.

Through wounds. The most common way of infection is through wounds; any form of wound will liberate the sap of the plant cell and establish a chemotactic focus, and in a sense it may be said that all infections are chemotactic. Roots, leaves and twigs may be broken by agricultural implements, by animals and other passers-by. It can easily be shown that tobacco plants can be grown in soil infected with *Pseudomonas solanacearum* provided their roots are sound, but plants whose roots have been pruned rapidly succumb to the Tobacco Wilt Disease. Hailstones may serve as wounding agents and the olive plantations of Italy have more than once suffered severely from attacks of Olive Knot caused by the entrance of *Pseudomonas savastanoi* (*Bacterium savastanoi* E.F.S.) after damage by hailstorms. By far the most usual cause of injury giving rise to bacterial infection is that caused by insects ; beetles, ants and aphids have all been known to produce such injury, and frequently have been found to carry the infecting parasite on their mouth parts.

Through natural openings of the plant. In most of the leaf and fruit infections the organisms gain entry through the stomata, establishing themselves in the sub-stomatal chamber and passing from there first into the intercellular spaces, and finally, after the vitality of the cells has been reduced by suffocation or poisoning, entering the cells of the plant.

Other unprotected openings are the lenticels and hydathodes. On any dewy morning the leaves of seedling cabbages can be found with a fringe of small glistening beads of water which have exuded from the hydathodes at the tips of the leaves. Any excess of water above the requirements of the tissue is, in the absence of free evaporation from the stomata, passed off in this way. Such droplets collect bacteria from the surface of the leaf, and if the weather be warm, may become fluid cultures with infective possibilities. Later in the day, when the evaporation rate has become more normal and the water requirement of the leaf tissue has increased, these droplets are drawn back into the hydathode, which, having no filtering mechanism, allows the bacteria to pass into the intercellular spaces of the leaf. It is so that *Pseudomonas campestris* usually commences its destructive mission in cabbages, turnips, &c.

Again, nectaries designed to attract bees and ants for the fertilization of the flower may become danger-points for the plant, if the attracted insect happens to be carrying on its proboscis, or its legs, an organism capable of attacking the plant. It is thus that *Bacillus amylovorus* gains access to pear and apple blossom. Infection spreads rapidly in this way ; in a few weeks a healthy and promising orchard may be reduced to a state which suggests that a fire has passed across it. From this feature the disease has received the name of Fire Blight.

The Defences of the Plant against Invasion by Bacteria.

The defence against bacteria put up by the plant is of an entirely different character from that put up by animals. There is in the plant no circulatory system comparable to the blood stream in animals, and, so far as present methods of research have enabled one to probe, there is no equivalent of the white cells of the blood ; whether phagocytic action may or may not take place within the protoplasm or some other constituent of the plant cell remains for the future to show.

The defences of the plant are:

Mechanical, i.e. structural features of the walls of the cells and the manner in which the cells are arranged in the formation of the tissue in the various parts of the plant. One of these outer defences is the cuticle, the chemical nature of which enables it to withstand well the solvent action of bacterial exudates. It is doubtful whether any living organism has the power to penetrate unbroken cuticle by means other than the employment of mechanical force. The cuticle is further guarded by waxy bloom and by hairs; both serve to protect the surface against the accumulation of water, which might otherwise collect in droplets and, becoming contaminated with bacteria, menace the tissue lying beyond the cuticle with mass action of bacteria or their products. Another barrier to the free entry of bacteria is found in the arrangement of the epidermal cells; these are set in such regular close formation as to leave no intercellular spaces; any bacterial penetration must, therefore, be by entry of the cells or by solution of the middle lamella.

Chemical. The constitution of the sap of the living cell must have a repellent action upon the majority of bacteria, for if inoculations of plants be made with organisms which have no pathogenicity for them, the organisms are unable to grow, and possibly may sometimes be killed immediately. This inhibition of growth cannot be attributed to lack of nutrient material since the sap of the same cells becomes eminently satisfactory as a culture fluid after the life of the cells has been destroyed by boiling or other means. There are very few genera of plants where bacterial disease is entirely unknown, but there is a considerable range of comparative resistance as between one family and another, and between one genus and another in the same family. The causes for such variation must be sought in the physico-chemical or the chemical nature of the sap. Speculation as to the nature of these repellent influences leads one to suspect first the acidic nature of the sap. Broadly speaking, plant pathogens can withstand higher hydrogen ion concentrations than the soil saprophytes, though it is doubtful whether the hydrogen ion concentration of plant sap is sufficiently high to be responsible for the complete inhibition of growth of the soil organisms. Assuming the contrary to hold, it would still be conceivable that saprophytes might differ from parasites in the action of their growth products in overcoming the degree of buffering possessed by the sap. Apart from acidity, other toxic materials are known to occur in plant saps, as, for instance, hydrocyanic acid generated on the disorganization of the cell from cyanophoric glucosides, essential oils, like mustard oil and cinnamon oil, menthol, thymol, camphor, &c. Plants which normally produce substances of such nature are certainly found to be among the least susceptible to bacterial attack. Special mention should be made of tannins since these are of such frequent occurrence in plants, and are generally believed to possess strong antiseptic properties.

It is probable, however, that it is not the tannins but their oxidation products which give the protection against parasitic invasion (Smith, 1911). Such oxidation arises from enzyme action at the time when the cell is thrown off its balance as the result of wounding or poisoning. The high tannin content of roots is probably a main factor in their defence. Defence here must be very efficient, otherwise it is difficult to conceive how plants manage to withstand the damage done to their roots by biting and gnawing insects.

The bark of trees seems particularly efficient in warding off bacterial attacks; here again it may be a virtue of the high tannin content of the cork cells. The formation of cork by meristematic plant tissue as a means of defence in preventing the further progression of a disease which has obtained a foothold in the tissue is a phenomenon with which the plant pathologist is very familiar. In these cases, and they are really very numerous, it can usually be shown that the production of tannin accompanies the rapid division of the cells and the suberization of their walls.

The question whether lysozymes occur generally in plant tissues has not received that attention from plant pathologists that its importance would seem to demand. Fleming (1922) discovered lysozymes in some plant juices, but found them to be considerably less effective than those found in certain animal secretions. A lytic substance has been isolated from rotting cabbage by Mallmann and Hemstreet (1924). Evidence of the presence of bacteriophage action in plants is also quite limited; Israilsky (1926) isolated a bacteriophage for *Bacillus tumefaciens* from tumours artificially produced on *Beta vulgaris* by inoculation with this organism. It was found impossible to recover *B. tumefaciens* from some one-day old cultures to which the bacteriophage had been added, and these authors ascribe the difficulty experienced in the isolation of the bacillus from the diseased tissue in Crown Gall to the presence of the bacteriophage.

Measures for the Control of Bacterial Disease of Plants.

Here it must be said at the outset that the available means of control are lamentably inadequate; in the greater number of cases, the only advice that the unfortunate grower can follow after his crop has been thoroughly ravaged by one of these bacterial pests is that for a space of four or five years that kind of crop should not again be carried on that land, the idea behind this advice being that if the pathogenic organism arose spontaneously from a perfectly innocuous forerunner it will during that time have an opportunity of returning to its normal saprophytic habit. Control measures are sometimes, however, more certain of effect. Cases are known in which the disease is carried by insects, where measures taken against these have proved quite effective. For example, the Cucumber Wilt is due to *Bacillus tracheiphilus*, and is carried mainly by the beetle *Diabrotica vittata*. Now this beetle has a special predilection for the wild-squash, and by planting rows of these between the cultivated cucumbers to act as a trap crop the beetles can be enticed on to them, where they can readily be dealt with by means of a kerosene spray. Where the carrying insects cannot be destroyed, as, for instance, bees and ants in the transmission of Pear Blight, the only thing to be done is to cut out the affected twigs and branches, taking every precaution to prevent spread of disease by the pruning-knife, and carefully collecting and burning the prunings. From the nature of the case it will be obvious that individual action on the part of a grower would be useless; pruning, to be successful, must be thorough and general over a large area. In the great fruit-growing districts of America, the orchardmen co-operate and sweep the orchards from end to end, but, even then, one learns, the efforts are attended with only partial success. The most promising treatment for bacterial diseases of plants in general lies in breeding varieties of plants which have been found to be of low susceptibility to such diseases; unfortunately, however, it has happened that the more resistant are less desirable in other respects. Some of the most choice varieties of hyacinths proved so susceptible to Wakker's disease, *Pseudomonas hyacinthi*, that they have disappeared altogether, and in Cobb's disease the best sugar-yielding canes have proved to be the easiest of attack by *Pseudomonas vascularum*. However, by selecting resistant varieties and crossing them with varieties bearing other desirable properties, much has already been accomplished, and the field is wide open for further efforts.

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CHAPTER VIII. *BACILLUS PESTIS* (SYN. *PASTEURELLA PESTIS*).

BY G. F. PETRIE (LISTER INSTITUTE, ELSTREE).

WITH A SECTION BY

W. BULLOCH (LONDON HOSPITAL).

History.

BY W. BULLOCH.

THE plague or pest has a long record in the annals of human suffering, and at various periods of history has been seen in pandemics of such magnitude as to constitute world events. To such belongs the pestilence which began in A.D. 542, in the fifteenth year of the reign of the Emperor Justinian, and lasted for the greater part of half a century. The historian Gibbon considered that a mortality of 100 million was a number 'not wholly inadmissible'. The 'Black Death' or 'Great Mortality' (1347–50) was also a world event, and it is computed by Hecker (1832) that the mortality was in the neighbourhood of 25 million human beings. Individual outbursts in particular towns have aroused great interest in the plagues of Milan (1630), London (1665), Marseilles (1721). This is not the place to deal with the history of plague epidemics, but those who wish to study the question will find it thoroughly dealt with in the writings of Hecker (1832), Hirsch (1881), Haeser (1882), Creighton (1891), Gasquet (1908), and particularly of G. Sticker (1908, 1910).

The modern pandemic of plague started in China, and its beginnings first attracted notice in Canton early in 1894. From Canton it was carried by steamer to Hong-Kong in May of the same year. In 1896 the disease appeared in Bombay, and was probably introduced by ship from some infected port. In the course of the next 20 years India became the scene of an appalling epidemic. R. B. Low (1920), who examined the mortality returns with great care, gave the reported plague deaths in India between 1896 and 1917 as 9,841,396, with a maximum in 1907 of 1,315,892—a rate of 5·16 per thousand of the population. During this period, 1894–1920, plague attained world-wide dissemination, concentrating itself, however, in particular places. It was in the Hong-Kong epidemic that the cause of plague—*Bacillus pestis*—was discovered. The Japanese Government sent Kitasato and Aoyama to study the disease. They arrived in Hong-Kong on 12th June, and on 14th June Aoyama made an autopsy. In the bubo, blood, spleen and other organs, Kitasato found a definite bacillus. From the spleen a mouse was inoculated. Kitasato also examined blood from a living case of plague and found bacilli showing polar staining and a distinct capsule. Cultures were readily obtained and were inoculated into mice, rabbits, guinea-pigs and pigeons. With the exception of pigeons all the animals succumbed and in their bodies were found the same bacilli which had been seen in man. The French bacteriologist Yersin, who arrived in Hong-Kong from Saigon on 15th June, 1894, also made investigations and found a bacillus similar to Kitasato's in most respects. Interesting details of the discovery of *B. pestis* have been published recently by Lagrange (1926) and Severn (1927).

It has generally been supposed that the bacilli of Kitasato and Yersin were one and the same, but Ogata (1897), Aoyama (1895), Yamagiwa (1897), and others have tried to make out that they were not. Kolle (1897) could find no essential difference. In the Indian epidemic bacteriological studies were carried out by Hankin and Leumann (1897) and Childe (1898), and as the epidemic increased in magnitude Commissions appointed by various European Governments proceeded to India, working especially in Bombay. The results of their investigations were published in a series of reports of which the most important were: the Austrian (Albrecht, Müller, Ghon and Poech, 1898–1900), the German (Gaffky, Pfeiffer, Sticker and Dieudonné, 1899), the British (Fraser, Wright and Ruffer, 1899–1901), and the Russian (Wyssokowitz and Zabolotny, 1897). At a later period the Advisory Committee appointed (1905) by the Secretary of State for India, the Royal Society and the Lister Institute published a long series of Reports (1906–1917) which did a great deal to further the knowledge of plague.

Apart from India and Hong-Kong the bacteriology of plague was studied in Formosa (Ogata, 1897; Yamagiwa, 1897), Alexandria (Gotschlich, 1900), New South Wales, 1900, 1902, 1903 (Thompson); Oporto (Calmette and Salimbeni, 1899; Kossel and Frosch, 1900), La Plata (Voges, 1902) and other places. A number of bacteriological studies of *B. pestis* were made, mostly in special plague laboratories, by Abel (1897), Hewlett (1897), Wilm (1897), Kolle (1897), Martini (1902), Kossel and Overbeck (1902), R. O. Neumann (1903), and many others.

Protective inoculation was carried out by W. M. Haffkine (1897) on an enormous scale in India. The production of plague serum was started by Yersin, Calmette and Borrel (1895). The pathological anatomy of plague was very thoroughly investigated by the Austrian Plague Commission and especially by H. Dürck, 1904.

The works referred to in the above paragraphs contain most of the facts on the bacteriology of *B. pestis*. The recent developments of the subject of plague have been especially concerned with its mode of transmission. A remarkable fact connected with the modern plague pandemic was noted in 1893, when the Chinese in Canton observed that a great mortality among rats preceded the outbreaks of plague in man. Abel (1901) has pointed out that the ancient literature, apart from a reference by Avicenna (tenth century), contains almost nothing certain about plague in animals. Haeser has cited a remark from Nicephorus (1348) that in the black death in Constantinople mice died. This is probably true, for the black (*M. rattus*) rat was unknown to the Greeks and Romans and first came to the shores of Europe in the Crusader's ships from the East. The common rat (*M. norvegicus; M. decumanus*) was a much later arrival and, indeed, was first noted in Copenhagen in 1716. Its great migration (Pallas, 1778) into Europe from the Caspian region was witnessed in 1727, when vast hordes crossed the Volga to invade Russia and finally to populate the world. There is a reference (Haeser, 1882) to rat mortality in the Pali plague in India in 1837, and the same was noted by C. Planck (1877) in plague in the North-Western provinces. From the time of the Canton epidemic plague in rats has been found in the majority of epidemics all over the world: in Hong-Kong (1894), Formosa (1896), Alexandria (1899), Kisiba (1898), Durban (1902), Odessa (1902), Oporto (1899), Naples (1901), and in S. America at Asuncíon (1899) and in Peru (1903). In some places spontaneous plague was found in other rodents such as tarbagans, ground squirrels and gerbilles, but the rat was the principal animal incriminated. Rat plague was also found in many ships from 1896 onwards. The mechanism of the transmission of the plague from rats to man was, however, doubtful for some time. By degrees it became apparent that the transmission occurs through fleas. This was first suggested by Ogata (1897), Simond (1898), and Gauthier and Raybaud (1903), but the flea theory was specially developed by W. G. Liston (1905), who showed that although certain species of fleas were as a rule restricted to particular species of hosts, they might also pass to other hosts, including man. Among such fleas he recognized *Pulex* (*Xenopsylla*) *cheopis* of Rothschild (1903). Liston's ideas were taken up and thoroughly investigated experimentally on a big scale by the workers for the Advisory Committee, among whom may be particularly mentioned G. Lamb, Petrie and Rowland. The actual means by which the plague fleas transmit the bacillus was worked out by A. W. Bacot and C. J. Martin (1914).

W. B.

General Bacteriology.

THE RELATION OF *B. PESTIS* TO OTHER BACTERIA.

The position of the plague bacillus in the scheme of bacterial classification has not yet been accurately defined but it is generally considered to be a member of the Pasteurella group (Winslow, *et al.*, 1920). With *B. pseudotuberculosis rodentium*, a kindred bacterium, it stands apart from the remaining members of the group, which cause hæmorrhagic septicæmia in the lower animals, by virtue of such cultural features as its ability to grow on media containing bile-salts, its inability to ferment saccharose, and the lack of production of indole and sulphuretted hydrogen. The plague bacillus resembles its congener so closely that cultural, serological, and cross-immunity tests have hitherto failed to afford any ready means of distinguishing between them. Thus MacConkey (1908) and Rowland (1912) succeeded in immunizing guinea-pigs against plague infection by means of cultures of the pseudotubercle bacillus. The first-named author obtained some degree of protection in rats against plague after immunization with a living culture of the pseudotubercle bacillus; on the other hand, Rowland failed to protect rats against a living virulent plague culture after he had immunized them with a chloroform-killed vaccine of the pseudotubercle bacillus.

The characteristics that aid in identification are as follows: First, the pseudotubercle bacillus is motile, an old observation which has been unaccountably overlooked until confirmed by Arkwright (1927); this is perhaps the chief distinction between it and the plague bacillus. Secondly, in litmus milk *B. pseudotuberculosis rodentium* produces alkali, whereas *B. pestis* produces no change. Lastly, white rats are susceptible to the pathogenic action of the plague bacillus but are immune against the pseudotubercle bacillus; this constitutes an important differential test.

THE MORPHOLOGY OF *B. PESTIS*.

In the Animal Body and in Cultures.

Smears of infected tissues when examined during life or after death show the plague bacillus as an oval rod with convex sides and somewhat rounded ends; its dimensions are from 1·5 to 1·75μ long, and from 0·5 to 0·7μ broad. The bacilli are mostly bipolar, and are disposed singly or as diplobacilli or in chains; they are generally extracellular. In artificial media the appearances are similar, but bipolar staining is not so well seen as in preparations from the tissues.

Pleomorphism: Involution Forms.

The plague bacillus presents a marked pleomorphism, and on occasion exhibits forms like cocci, streptococci, streptothriciæ, and even mould and yeast forms; feebly staining bladder or ring forms are also seen, especially in the primary bubo. These so-called involution forms are important from the point of view of diagnosis. The general belief is that they are not degeneration forms, because they yield normal cultures, which are capable of infecting susceptible animals. The Plague Research Commission in India (1907)* noted that on rare occasions the plague bacillus in the organs of rats found dead of acute plague resembled a small cocco-bacillus like the bacillus of fowl cholera. According to the same authority (1910), the abscesses in the spleen that are characteristic of the visceral type of resolving (chronic) plague in the rat occasionally contained involution forms, which were sometimes very scanty and were rarely numerous ; the necrosed areas of similar origin in the spleen also sometimes showed them.

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* Throughout this chapter the term 'Plague Research Commission in India', or briefly, 'Plague Research Commission', or, where the context permits, 'The Commission', refers to the working Commission in India which was appointed in 1905 by an Advisory Committee representing the Secretary of State for India, the Royal Society, and the Lister Institute.

Yeast-like forms of *B. pestis* were seen by Bacot (1915[1]) in smears of the stomach contents of bugs ; they were never a distinctive feature in the stomach of infected rat-fleas. Cornwall and Menon (1917) observed peculiar protozoon-like involution or developmental forms in experimentally infected bugs.

Albrecht and Ghon, the pathologists of the Austrian Plague Commission (1898), stated that 5 per cent. glycerin agar and a medium containing 5 per cent. of sugar favoured the appearance of polymorphous forms of the plague bacillus. Hankin and Leumann (1897) discovered that the addition to agar of 3 per cent. sodium chloride produces a striking variety of bizarre forms which are of diagnostic value. These observations on the effect of high concentrations of glycerin, sugar, and salt may signify that the presence and characteristic shape of polymorphous forms have some relation to the osmotic pressure of the medium.

Branching forms of the plague bacillus have been described by the Austrian Plague Commission and others, but whether such forms really exist or not is uncertain.

Staining Reactions.

The plague bacillus is a Gram-negative bacterium. To obtain good bipolar staining, preparations from tissues or cultures are best dried in the air, and then treated with absolute alcohol or an alcohol-ether mixture for one minute ; the fluid is poured off, and the remainder got rid of by warming or burning in the flame. Dilute carbol-fuchsin, carbol-thionin and Loeffler's methylene blue are suitable stains. For staining plague bacilli in sections the Unna-Pappenheim methyl-green-pyronin stain is very satisfactory and Ehrlich's hæmatoxylin and orange-rubin may also be used. The tissues can be fixed in corrosive sublimate solution, or alcohol or Orth's fluid.

The Capsule of the Plague Bacillus.

Rowland (1914) confirmed the work of previous observers who stated that the plague bacillus possesses a capsule. He distinguished between a typical capsule and an envelope which, as seen in an Indian ink preparation, has no definite outer limit. The envelope is probably viscid, and may contribute to the sliminess of plague cultures on solid media ; it is insoluble in water but readily soluble in dilute alkali ; and it is best seen in broth to which 10 per cent. of heated serum has been added, when a culture in this medium is incubated at a temperature of 36° C. The typical capsule of the plague bacillus has a sharp regular outline, and is distinctly visible in unstained specimens; it is coloured by the usual capsule stains. The presence of capsules is inconstant, and thus they may be difficult to demonstrate. Rowland found them in experimentally infected rats, but only at the site of inoculation, and in cultures that were grown in a serum medium; when the bacilli were grown in horse serum, which has the power of lysing certain strains, he sometimes saw many of them with a definite capsule.

The Question of Motility.

In his original description of the plague bacillus Kitasato (1894) stated that it showed a very indolent motility. A few other workers, including Gordon (1897), also convinced themselves that they observed motility. Most workers have never seen motile plague bacilli, nor have they been able to demonstrate flagella (Albrecht and Ghon, 1898; the German Commission, 1899; and Kossel and Overbeck, 1902). In a recent paper Arkwright (1927) confirms the statement of some early observers that *B. pseudotuberculosis rodentium* is a motile organism, especially when grown at room temperature. He ascertained that a homologous serum agglutinated this bacillus according to the rule for motile bacteria, because suspensions, when heated to 56° C. for 20 minutes, gave loose flocculi to the limit of the titre, but not after heating to 100° C. for 1 hour. Suspensions of *B. pestis* after being heated at 100° C. were likewise no longer agglutinable by this serum. Arkwright remarks that in view of the positive findings of Kitasato and of Gordon it is interesting to know that the agglutination of *B. pestis* by a pseudotubercle serum is due to a heat-labile substance such as is usually associated with flagella. Nevertheless, he has not observed motility in cultures of the plague bacillus.

THE CULTIVATION OF *B. PESTIS*.

The Conditions for Growth: Temperature, Reaction of the Medium, Moisture, and Oxygen Tension.

The temperature range for growth of the plague bacillus is from 4 to 43° C., with an optimum at about 30° C. Growth takes place best in a neutral or weakly alkaline medium. Six old laboratory strains of *B. pestis* examined by d'Aunoy (1923) showed a range of growth from pH 5·0 to 8·2 in salt-free veal broth; optimum growth took place from pH 6·2 to 7·0. After passage through white rats the same strains showed a range of growth from pH 5·4 to 7·6 with an optimum between 6·0 and 6·6. To obtain the characteristic slimy growth of *B. pestis*, solid media on which it is grown should be moist.

Most authors stress the necessity for free access of the cultures to air. Albrecht and Ghon tested 20 strains and obtained good growth in sugar-agar stabs; they, therefore, considered the bacillus to be a facultative anaerobe. Balfour Stewart (1900) thought that the plague bacillus did not need a large supply of oxygen, because an anaerobic culture was quite as virulent as a control culture grown aerobically. Drennan and Teague (1917) made the interesting observation that when plague bacilli are sown sparsely on agar plates, growth may not take place, but that a heavy inoculation may produce luxuriant growth; Schütze has since made similar observations (unpublished). The American authors discovered also that the addition of sodium sulphite to agar favoured the surface growth of the plague bacillus. They advance various explanations, but do not mention the one that is most probable, at least in the view of the present writer, that their observations may indicate the favouring influence upon growth of an optimal oxidation-reduction potential brought about by the reducing action of the sodium sulphite, and by reducing substances in the bacillary inoculum.

The Growth Characteristics of the Plague Bacillus on Various Media.

Surface and deep growth on agar and gelatin.

Early growths on dry agar when looked at by reflected light resemble a ground-glass surface. After 48 hours, two kinds of colonies are distinguishable. These are described by Albrecht and Ghon as follows: Type I is a roundish, sharply-defined, raised colony with steep edges, grey-white in reflected light, and bluish in transmitted light. Type II is a larger colony than type I with a central nucleus and a more or less broad, delicate peripheral zone with wavy edges; the central part may be poorly developed, so that the colonies appear uniformly flat and delicate. Yersin (1894) observed that some of the colonies grew better and more quickly than the others, with the result that discrete dense colonies projected above the delicate surface layer of a pure culture. Secondary colonies of this kind may grow to a considerable size—the so-called giant colonies; for the most part they belong to type II, and are apt to be mistaken for contaminations. If a culture, a month old, is examined, the type II colonies will be seen to show fine dewdrop-like papillæ scattered over the peripheral zone, which give to the colony a distinctly granular look (Plague Research Commission, 1908). Eastwood and Griffith (1914) also state that minute papillæ are sometimes visible in the peripheral zone of the parent colony.

Tuft-like outgrowths from the central line of growth often appear in old stab-agar and stab-gelatin cultures. A surface film of growth may or may not form.

Growth in broth.

Flocculi appear on the sides and bottom of the tube, and a surface film of growth forms later with fine thread-like strands depending from it; these are miniature stalactites. The broth itself is generally clear, but varying degrees of turbidity may be present, and are related to the conditions of growth and to the strain of culture used. In the writer's experience the growth of the plague bacillus in broth never exhibits the uniform turbidity which is characteristic of *B. pseudotuberculosis rodentium*, and which is probably related to its motility.

Special cultural features.

Change in reaction. In the course of growth, broth cultures become alkaline. Naidu and Jang (1927[1]) estimated the initial hydrogen-ion concentration of 100 different brews of broth used for the preparation of

Haffkine's prophylactic fluid, and found it to be mostly between pH 6·6 and 7·0. They investigated the changes of pH during growth in this medium, and noted a gradual increase in the amount of alkali produced from week to week, with a maximum between the fifth and eleventh weeks (1927[2]).

TABLE I.

Showing the change in pH produced in broth cultures of 58 strains of *B. pestis*.

Group of strains.	No. of strains.	Cultivation period.	Incubation temperature.	Initial pH.	Final pH.
A	5	9 weeks	6° C.–12° C.	7·3	7·8
B	53	7 weeks	10° C.	7·4	8·4
C	6	8 weeks	11° C.–15° C.	7·6	8·0

Table I shows the change in reaction brought about in 64 broth cultures that were grown for the purpose of obtaining plague toxins ; 58 strains of *B. pestis* were used. Each group of strains was grown in the same batch of medium. Group B, consisting of 53 strains, was grown in broth which contained 1 per cent. of horse serum ; groups A and C were grown in plain broth. The series of 53 strains produced the greatest change in reaction, namely from pH 7·4 to 8·4 (Petrie : unpublished observations).

Sliminess of cultures. As already stated, this character is perhaps attributable to the envelope round the bacillus described by Rowland. The factors influencing sliminess of cultures appear to be the temperature of incubation, the moisture of the medium and its reaction, and growth in the animal body. Thus Horiuchi (Dieudonné and Otto, 1927) found that cultures grown at 37° C. are more slimy than those grown below 25° C., and further that non-slimy strains recover the character by growth in the animal tissues. According to Segawa (Dieudonné and Otto, 1927) the more alkaline the medium the less slimy is the culture. MacConkey (1908) thought that sliminess was more characteristic of the plague bacillus than of the pseudotubercle bacillus. Strong and Teague (1912) concluded that plague cultures became more slimy as the temperature of growth decreased from 37° C. to that of the ice-chest, that freshly isolated strains were more viscid than old laboratory cultures grown on agar, and that an agar medium which contained glucose or saccharose or starch did not give more slimy cultures than a sugar-free agar medium, when grown at 32° C.

Stalactites. The Report of the Plague Research Commission (1908) contains data on the formation of stalactites. Absolute lack of vibration is the first essential in their production. The addition of oil to the broth is an advantage, but good stalactites can be obtained without its use. Broth with a typical growth of stalactites is clear. Cultures fresh from the body may not readily form them ; in an instance given by the Commission stalactites appeared for the first time on the eighth day ; and occasionally the cultures fail altogether to form them.

The Maintenance and Viability of Stock Cultures.

Stock cultures live well in agar stabs kept in the dark at room temperature ; they may remain alive for months and even years.

Lysis in Plague Cultures.

Autolysis. The term ' autolysis ' denotes an escape of the water-soluble protein of the plague bacillus into the culture medium brought about by a process of maceration of the bodies of the bacilli after their death. It also implies a subsequent breaking down of the protein by means of the proteolytic enzyme which is set free at the same time. In a 4-day broth culture tested by Rowland (1910) no coagulable protein was present, but after 14 days there was a considerable amount ; in one test there were 14 mgm. per 100 c.cm. of culture. Autolysis goes on progressively in broth cultures that are incubated for several weeks. The increasing toxicity of the broth due to the soaking-out of the endotoxin is an index of the degree of autolysis.

Lysis by means of a ' phage '. d'Herelle (1921 and 1925) obtained a bacteriophage from the fæces of rats that were caught in four different places in a town in Cochin-China in which there was a small outbreak of human plague ; these rats had presumably resisted the infection, and a very active bacteriophage was obtained from all the four sources. When this strain of bacteriophage had undergone about 100 passages 0·02 c.cm. of the ' phage ' culture completely lysed 10 c.cm. of a 24-hour broth culture of *B. pestis* after incubation for 20 hours at 37° C. There is no mention in his account of the examination of control rats from a neighbouring uninfected locality for the presence or absence of ' phage '.

Mutation.

Markl (1914) studied the types of colonies that grow on agar plates with the object of ascertaining whether they are true mutants or not. His account of three types of colonies that he observed on plating agrees with the usual descriptions. Subcultivations of these types showed that they possessed no fixity of character ; moreover, all were of the same degree of virulence. Markl, therefore, does not consider that they are true mutants.

THE BIOCHEMICAL REACTIONS OF *B. PESTIS*.

Its Action on Carbohydrates.

The plague bacillus ferments a number of carbohydrates with the production of acid but not of gas. An analysis of the work of 12 investigators (Table II) shows that glucose and lævulose are invariably fermented, whereas lactose, saccharose and raffinose invariably undergo no change. Maltose, mannitol and galactose are usually fermented ; dulcitol and inulin usually undergo no change. Wade (1916) suggests

TABLE II

S arizing th en n r ions of *B. pes* rded 2 sets o ons.

Observer.	Glucose.	Lævulose.	Mannitol.	Galactose.	Maltose.	Inulin.	Dulcitol.	Lactose.	Saccharose.	Raffinose.	Adonitol.	Amygdalin.	Arabinose.	Dextrin.	Glycerin.	Inositol.	Isodulcitol.	Nutrose.	Quercitol.	Rhamnose.	Salicin.	Sorbitol.	Sorbose.	Starch.	Xylose.	Litmus Milk.
MacConkey (1905, 1908)	+	+	+	+	+	o	o	o	o	o	o	o		+								o				o
Plague Research Commission in India (1908)	+	+	+	+	+		o	o	o					+												
Wherry, W. B. (cited by McCoy, G.W. 1909^{1}).	+	+	+	+	+	o		o	o																	
Castellani & Philip (1914)	+	+	+	+	+		o	o	o																	
Eastwood & Griffith (1914)	+	+	+	+	+		o	o	o	o	Slow 12 days. +				o		Usually o. If + very slow				Slow 6 days. +					o
Swellengrebel & Hoesen (1915^{2})	+	+	+	+	+	+	+	o	o				+	+	o		o			+			+		+	
Wade .. .. (1916)	+	+	+	Usually +	Usually +	o	o	o	o	o	o	o	Usually +	o	3 in 10 +	o		o			Usually +	o				
Philip & Hirst .. (1917)	+	+	+	+	+	o	o	o	o	o			+	+	o		o				+	o				
d'Aunoy .. .. (1923)	+	+	+	+	Usually +	o	o	o	o	o	o		Most +	o	Some +				o		Some +	o			o	
Pons, R. .. .. (1925)	+		Usually +		+			o	o																	
Berlin (cited in Dieudonné & Otto 1927)	+	+	+	+	+	o	o	o	o	o	o		+	o										o		
Pirie .. .. (1927)	+	+	+	+	+			o	o						4 in 23						21 in 23 +					

that the lack of agreement in the results of some workers is due to technical differences, and not to the existence of fermentative varieties, because each worker obtains consistent reactions with different cultures from various sources. He could find no evidence for any modification of fermentative power after prolonged artificial cultivation. The fermentations were more active at room temperature than at 37° C. Cultures grown anaerobically at 37° C. fermented more actively than those grown aerobically at room temperature. He considers that the titration of sugar broth is by far the best way of determining the degree of acidity due to fermentation, and that Hiss's serum-water media give misleading results. The Plague Research Commission (1908) found that an avirulent strain, isolated probably seven years before, gave reactions similar to those of fresh virulent strains. Gordon (personal communication to Petrie and Macalister, 1911) ascertained that the pseudotubercle bacillus readily ferments isodulcitol and glycerin with acid production, and that the plague bacillus has no effect on these substances. The distinction is, however, not absolute (Petrie and Macalister, 1911 ; Eastwood and Griffith, 1914). Thus the last-named authors found that of 35 strains from plague-infected rats, 6 fermented isodulcitol after an interval varying from 20 to 40 days ; 29 strains gave no change. Three out of 10 of Wade's strains of the plague bacillus fermented glycerin, and some of d'Aunoy's strains were also positive fermenters. Of 23 South African strains of plague examined by Pirie (1927), 4 (1 human and 3 rodent strains) were glycerin fermenters ; these were all from the same locality, namely the North-West of the Cape Province, where in the middle of 1926 plague broke out for the first time. The fermentation test with salicin is erratic. Twenty-one of the 23 South African strains fermented salicin, but Wade and also d'Aunoy obtained less constant results with salicin than with some of the other carbohydrates they tested. Eastwood and Griffith noted that the fermentation was slow, with a latent interval of about 6 days.

Indole Production : Action on Gelatin and Milk.

There is general agreement that indole is not produced. Gelatin is not liquefied. The evidence is in favour of the absence of acid production and clotting in milk ; Eastwood and Griffith observed no change when 35 rat strains were grown for 28 days in litmus milk.

The Acid Agglutination Test for the Plague Bacillus.

The acid agglutination test for bacteria, which was introduced by L. Michaelis, was applied to the plague bacillus and its allies by Stepanoff-Grigorieff (1913) and by Markl (1915). When the results of the first-named author are converted into terms of pH we find that 4·4 or 4·2 is the optimum hydrogen-ion concentration for precipitation of the plague bacillus. Markl found the optimum reaction for the plague bacillus to be at pH 4·1 and 3·8 ; he believes that the test is useful for the purpose of identification.

THE BEHAVIOUR OF *B. PESTIS* IN CERTAIN ENVIRONMENTAL CONDITIONS.

Attention in the past was focussed upon the ability of *B. pestis* to resist destructive agencies, and many observations were made to estimate the duration of its life when it was submitted to such physical conditions as light, heat, cold and desiccation. The methods of experiment were necessarily rather artificial, and were influenced by the assumption that the saprophytic life of the plague bacillus was of great importance in the complex of circumstances that help to spread the infection. The investigations of the Plague Research Commission in India (1906–17) completely overturned this hypothesis by showing that the strictly parasitic life of the bacillus in its insect and mammalian hosts is the outstanding factor in the spread of plague. Hence, only a brief account will be given below of the viability of *B. pestis* under various external conditions.

Light. The plague bacillus is easily killed by exposure to sunlight. Rowland (1915) found that ultra-violet light was bactericidal, and that the active region of the available spectrum lay between the lines 237 and 296$\mu\mu$; five minutes' exposure to the radiation from a carbon arc killed the bacilli. Galeotti (1916) and his co-workers Schiavone and Trerotoli (1914), used a 75-volt mercury vapour quartz lamp, and exposed in watch-glasses suspensions which were not more than 2 or 3 mm. deep at a distance of about 20 cm., and at a temperature not higher than 30° C. The suspensions were prepared in saline, broth, urine, milk and blood-serum. The time of destruction of the bacilli in these liquids increased in the order just given from about 8 minutes in saline to more than 3 hours in blood-serum.

Dry and moist heat. Exposure for 1 hour to dry heat at 100° C. kills the bacilli when they are dried on slides (Abel, 1897). To sterilize broth cultures or bacillary suspensions German workers consider it necessary to heat for 1 hour at 65° C., and Kolle (Dieudonné and Otto, 1927) thinks it is an advantage to shake the cultures during the heating process in order to disperse the bacterial clumps and to maintain a more uniform temperature. The workers at the Haffkine Institute in Bombay sterilize broth cultures destined for use as a vaccine by heating them for 15 minutes at an average temperature of 55° C.

Cold. The plague bacillus is not very sensitive to the action of cold ; daily freezing and thawing for 40 days did not kill it (Gladin, 1898), and Kasanski (1898) found it alive after having been kept for five and a half months at a temperature of —31° C. According to Schurupoff (1912) bacilli in the frozen plague corpses in Manchuria were still viable after the lapse of a year.

Desiccation. The bacilli are quickly killed by drying over sulphuric acid at about 30° C. ; the higher the temperature during the drying process the shorter is the period of survival. Experiments have shown that plague bacilli in inflammatory exudates and in sputum remain alive for a considerable time, because they are in contact with substances such as mucus and proteins that protect them from desiccation. The German Commission (1899) found that the bacilli when dried on wool, silk, linen, glass or wood, lived for six days at the most.

The influence of associated bacteria. The plague bacillus does not thrive well in company with other bacteria, especially at temperatures between 30° C. and 37° C. In living tissues it is soon overgrown by associated organisms such as the pyogenic cocci, and in the organs after death it is gradually swamped by the advancing tide of putrefactive bacteria.

The action of disinfectants. The plague bacillus possesses little resistance to the action of disinfectants.

General conclusion. The plague bacillus has feeble powers of resistance to adverse influences, and it is, therefore, not well adapted to a saprophytic life.

THE PATHOGENIC ACTION OF *B. PESTIS*.

Experimental Plague in the White Rat.

A subcutaneous injection with cultures or suspensions of infected tissues generally causes death within seven days. Post-mortem examination reveals swollen lymphatic glands near the site of injection, which are surrounded by œdematous and hæmorrhagic tissue and which contain many plague bacilli. The appearance of the spleen varies ; either it shows nothing abnormal or it is enlarged, and firm and dry on section. In an animal which has survived longer than a week grey foci may be scattered over the surface of the spleen. The liver in acute cases is congested ; at a later stage necrotic foci may appear. The lungs are often congested. Eastwood and Griffith (1914) inoculated tame rats with plague-infected tissues from the naturally infected *M. norvegicus* that were found in East Anglia ; the general redness and vascular engorgement throughout the ventral subcutaneous tissues were constant and conspicuous features. The experimental disease in the rat is characteristically a septicæmia, but, when the inoculated strain is specially toxigenic, the bacilli in the organs may be scanty, even although they are abundant in the primary group of glands.

According to the experience of Kolle (1901), the cutaneous method of Albrecht and Ghon does not produce a fatal infection in tame rats with the same certainty as in guinea-pigs. Douglas, on the contrary, found that in 167 experiments only 3 per cent. of the rats survived (Bulloch and Douglas, 1909). In Rowland's view the subcutaneous method gave more constant results in white rats than the cutaneous method, and he, therefore, adopted it in his work. The intraperitoneal method causes a rapidly fatal septicæmia, but if an excessive dose of a culture is given by this route, death seems to be due to a toxæmia, because comparatively few bacilli are recognizable in the internal organs. An exaggerated importance was formerly attached to infection by the mouth, since it was thought that in nature this is a common mode of infection among rats. Kolle (1901) infected 60 rats by feeding, with the result that a neck bubo was present in 40 ; in 4 there was a primary plague pneumonia ; and only in 2 were primary mesenteric buboes seen.

Inhalation experiments on rats carried out by Martini (1901) killed 32 out of 36 animals in 3 or 4 days with lobular and sometimes with lobar lesions in the lungs. The lesions contained numerous plague bacilli, in contrast with the scanty numbers that were present in the heart-blood and spleen; this particular distribution of the bacilli strongly indicates the existence of a primary plague pneumonia. Plague bacilli may pass through the uninjured nasal and conjunctival mucous membranes ; a cervical bubo appears, and the death of the rat takes place within a few days. According to Bandi (1899) the pneumonia that is apt to follow infection by the nasal route is secondary to the cervical bubo, and is not, as Batzaroff (1899) thought, a primary plague pneumonia. The bacilli probably pass along the nasal duct to the lymphatic tissues in the throat, and so give rise to the cervical bubo.

Chronic plague lesions in rats. Chronic plague lesions in rats have been described by Kolle and Martini (1902), Albrecht and Ghon, and Hata (1904). They were formerly considered to be of importance as a possible reservoir of the infection in the interval between epizootics. The lesions found by these workers were cheesy bronchial glands, solid induration of the lungs, and encapsulated foci in the submaxillary glands ; they do not correspond with the descriptions given by the Plague Research Commission in India (1906, 1907, 1910, 1912) of naturally occurring resolving (chronic) lesions in rats.

Experimental Plague in the Guinea-pig.

The appearances in the guinea-pig after subcutaneous inoculation resemble those found in rats, but there are one or two special features. A general subcutaneous œdema is characteristic of experimentally infected guinea-pigs ; and the organs, and especially the spleen, liver, and lungs, are often studded with pseudotubercle-like nodules. Eastwood and Griffith noted that the lungs were usually congested and œdematous, and that they showed in places consolidated areas. They were frequently studded with soft greyish-white nodules, which exhibited a congested periphery ; the largest of the nodules were found in the less rapidly fatal cases, and in one animal that had lived for 10 days, they had a diameter of 5 to 6 mm. ; smears of the lung nodules showed many plague bacilli.

A modification of the subcutaneous method of inoculation is the expedient of inserting a small piece of infected tissue into a skin-pouch (Hauttasche). The cutaneous method is performed by rubbing the inoculum into an abraded surface about one inch square on a guinea-pig's abdomen ; plague bacilli are able to penetrate through even the intact skin, and the abrasions of the skin naturally assist their entry.

The Plague Research Commission (1907) inoculated cutaneously and subcutaneously a parallel series of guinea-pigs with graduated quantities of a fresh virulent human strain, ranging from 1/40 to 1/4,000,000 of a cubic centimetre. The guinea-pigs that were given the two smallest doses cutaneously recovered, whereas the corresponding subcutaneously inoculated animals acquired a chronic form of plague. When a guinea-pig is inoculated cutaneously the local reaction may be seen as early as 12 hours after the inoculation ; it consists of phlyctenules surrounded by a zone of redness ; inguinal and sometimes axillary buboes appear and can then be felt. In more chronic cases the glands are palpable only after an interval of several days. An early decrease in weight is a valuable sign that a plague infection is in progress.

Swellengrebel and Otten (1914) described a variety of plague in experimentally infected rats and guinea-pigs which they term 'mitigated plague', but which appears to be a form of subacute plague. It is associated with a partial immunity in experimental rats and follows a minute dose of plague bacilli when given cutaneously to guinea-pigs. The authors surmise that the mitigated form may occur naturally in rats, and that it may pass into the acute form after a shorter or a longer time, but the evidence for this seems to be slender.

The intraperitoneal injection of guinea-pigs causes rapid death, generally in 24 hours after the injection, with a purulent peritonitis and septicæmia. Ohoto (1924) cultivated 0·6 c.cm. of blood obtained by cardiac puncture from 9 guinea-pigs, 3 of which were inoculated cutaneously, 3 subcutaneously, and 3 intraperitoneally. The culture was positive in all when the sample of blood was taken just before death. After cutaneous inoculation it was positive within 72 hours ; within 34 hours after subcutaneous inoculation ; and within 24 hours after intraperitoneal inoculation.

Experimental Plague in the Mouse.

White rats and guinea-pigs possess an almost uniform susceptibility to plague, mice are less susceptible, and rabbits are least susceptible to the infection. According to the German Commission an occasional mouse resists subcutaneous infection with a virulent culture without showing symptoms of illness. Irregular results are still more noticeable if cultures of low virulence are used. Kolle and Martini (1902) observed that if mice are inoculated with weakly virulent cultures they remain apparently unaffected, but die of plague sepsis after 10 to 20 days. Mice are susceptible to the usual modes of infection, but the results are inconstant.

Experimental Plague in other Animals.

Cats are not very susceptible, and dogs are resistant. The bat (*Vesperugo noctula*) was found by Gosio (1902) in Naples to be susceptible ; and Leger and Baury (1923) have found the African bat (*Nyctinomus pumilus*) to be very susceptible to plague ; indeed they suggest its use as an experimental animal in colonial laboratories where white mice are difficult to obtain. Monkeys are easily infected with plague ; the grey monkey (*Semnopithecus entellus*) is much more susceptible than the brown (*Macacus radiatus*). The camel, which is used as a beast of burden in the Kirghese steppes in Southern Russia, is stated by Nikanarov (1926) to be susceptible to plague.

Experiments have been made in various parts of the world to test the accuracy of the conclusion drawn by early workers in Hong-Kong that domestic animals are susceptible to plague. A detailed account of these experiments is unnecessary, because they are mutually confirmatory; they give no support to the original statement.

Although *M. rattus* and *M. norvegicus* remain the most important members of the Rodentia that convey plague to man, the list of rodents associated with human plague has been extended within recent years. In contrast with the rat, or at least *M. rattus*, which is typically a house-rat, these rodents live in the open country: the tarbagan in the steppes of the Far East, the ground-squirrel in California, and a variety of field rodents on the veld of South Africa. They can be infected with plague under experimental conditions.

Problems in Plague Immunity.

THE VIRULENCE OF THE PLAGUE BACILLUS.

The normal virulence of the plague bacillus, when tested on experimental animals, is of a high order. Barber (1912) found that 6 out of 9 guinea-pigs and 2 out of 12 monkeys that received one virulent bacillus died of plague. Freshly isolated strains are invariably virulent (Plague Research Commission, 1907; Eastwood and Griffith, 1914). The virulence of the bacillus in cadavers at moderate temperatures is unimpaired by association with putrefactive bacteria. Of 152 laboratory cultures of diverse origin, some of which were many years old, only 12 proved to be avirulent or showed a notable loss of virulence; the remainder—92 per cent.—were virulent (Albrecht and Ghon, 31 strains; Otto [Dieudonné and Otto, 1927], 45 strains; McCoy, 1909[1], 8 strains; McCoy and Chapin, 1912, 68 strains). The virulence is not readily attenuated by prolonged cultivation, and it may be retained for years if the culture is kept moist in the dark at a low temperature. The addition to the medium of some kind of protein is useful in helping to conserve and enhance virulence. Experiments made by Rowland (1914) with various proteins (egg-white, pure crystalline egg-albumen, horse serum, and ox-spleen juice) indicated that for full virulence the plague bacillus needs a protein diet; he had already shown that it contains a proteolytic enzyme.

A culture which is avirulent for the rat may still be pathogenic for the guinea-pig (Kolle, Hetsch and Otto, 1904). Strains that have lost a part of their virulence may regain it by passage through susceptible animals, but some strains fail to respond to this treatment. Passage through guinea-pigs and rats, without intervening culture on artificial media, does not alter the original virulence of a plague strain either for the animal used for the passage or for other experimental animals (Albrecht and Ghon, Otto, Plague Research Commission).

Many attempts have been made to reduce the virulence of the plague bacillus by growing it in unfavourable conditions, for example, from 40 C. to 45° C., or by adding carbolic acid and a variety of dyes to the medium, but none has proved successful except perhaps the method used by Hetsch (Kolle, Hetsch and Otto, 1904) of growing the culture in broth which contains increasing concentrations of alcohol. Burgess (1927) reduced the virulence of a strain by inoculating it into a wild rat (*Cricetomys gambianus*) that had been rendered partly immune by means of a vaccine. The resulting loss of virulence brought about a five-fold increase in the survival period of experimentally infected animals ; the virulence was not restored by passage.

As will appear later, the virulence of a strain has no relation to the toxicity of the bacterial substance, but is rather an expression of its capacity to multiply in the tissues of the host. There is no standard method of testing for virulence. The method of Kolle and Martini (1902) consists of rubbing a test-dose of the culture, for example, one-fifth of a loop in 0·2 c.cm. of broth, on a carefully shaved area of standard size on a guinea-pig's abdomen. If the culture is avirulent, the guinea-pig recovers with at most a moderate glandular swelling ; fully virulent cultures kill in 4 to 5 days ; and cultures of intermediate virulence produce proportional effects. Kolle and Krumbein (1909) advise dipping a syringe needle of a standard calibre in a bacterial suspension of a definite concentration, and introducing the charged needle under the skin near the root of the tail of a rat. Rowland (1910) actuated the piston of his syringe by means of a screw so arranged that one turn caused delivery of 0·1 c.cm. of the culture. The needle was connected with the nozzle of the syringe by pressure tubing and was pushed through the skin and muscles of the left thigh of the rat until the point reached the subcutaneous tissue on the other side of the thigh, where the inoculation was made. On withdrawing the needle the closure of the track in the muscles prevented regurgitation of the inoculum.

THE ENDOTOXIN AND THE IMMUNIZING SUBSTANCE OF THE PLAGUE BACILLUS.

The Significance of The Terms ' Endotoxin ', ' Antigen ', ' Vaccine ' and ' Immunizing Substance '.

There is general agreement that the toxin of the plague bacillus is an endotoxin which is set free as the result of disintegration of the bacillary bodies in old broth cultures, or by extracting the culture-mass obtained from surface growths on agar. The terms antigen, vaccine, endotoxin and immunizing substance have been used by workers on the subject in no very strict sense, and almost as if they were interchangeable ; the reason has doubtless been that up to the present it has not been possible to effect a separation of the constituent antigens in solutions of bacterial protein. There is no ground for believing that the antigenic substances that are present in old autolysed broth cultures of the plague bacillus are in any way different from those obtained by extracting the bacillary bodies, although naturally the extracts represent a purer form of the essential products of the bacillus, since they are free from the nutritive substances of the culture medium. And again, 'whole' vaccines such as Haffkine's vaccine and bacillary suspensions are not essentially different from the preparations just mentioned. Like broth toxins, they contain, in addition to the antigen, non-essential constituents which have no specific immunizing value. In the account that follows, the behaviour of the various products of the plague bacillus is discussed without regard to their mode of preparation.

Methods of Preparing the Toxin and Immunizing Substance.

'Whole' vaccines.

Haffkine's vaccine. In the Haffkine Institute, Bombay, this vaccine is prepared by growing a virulent strain in flasks of broth for from 6 to 8 weeks at room temperature (23 to 29° C.) in the dark, the depth of the medium being such as to ensure the maximum surface. The culture medium is made by digesting goats' flesh or wheat flour with hydrochloric acid for 3 days at a temperature of 70° C., neutralizing with soda, filtering the fluid, and making it slightly alkaline to litmus. The initial reaction lies with great constancy between the limits pH 6·6 to 7·2 (Morison, Naidu and Avari, 1924[1]). After the requisite incubation period the culture is sterilized by placing it for 15 minutes in a water-bath at an average temperature of 55° C. After the addition of 0·5 per cent. phenol, the vaccine is then filled into ampoules and sterility tests are carried out. The dose for an adult is 3 to 4 c.cm. Reference to the application of this vaccine to human prophylaxis will be found on p. 204.

Heated bacillary suspensions. The German Commission removed the bacilli from cultures grown on agar for 48 hours, and suspended them in broth or saline; fresh virulent cultures were used. The suspension was heated for from 1 to 2 hours at 65° C., and 0·5 per cent. phenol was afterwards added. A quantity of the vaccine equivalent to one agar-slope culture represented a dose for an adult. Elsewhere than in India this type of vaccine is preferred.

The Lister Institute vaccine. At the Lister Institute the method of preparation follows indications given by Rowland's work on plague vaccines. A body-strain of *B. pestis* is grown in broth at 28° C. for 3 days; a tenfold dilution of the resulting culture is made in heated horse serum, and 5 c.cm. of the mixture are used as seed for each agar Roux bottle. After 3 days' incubation at 28° C. the agar growths are washed down with 0·75 per cent. phenol-saline, pipetted off, counted, and allowed to stand at room temperature until the suspension is found to be sterile. A vaccine of a standard strength of 3,000 million bacilli per c.cm. is prepared in 0·5 per cent. phenol-saline. The dosage recommended is 1,500 million bacilli, followed after 7 days by 3,000 million bacilli (Schütze, 1925).

Broth toxins.

Markl (1898, 1901) was the first worker to give an adequate account of the conditions necessary for obtaining a toxin in filtered broth cultures. G. Dean (1906), Rowland (1910) and MacConkey (1912) found toxin production in broth cultures to be quite fortuitous, but the present writer (unpublished work), by following Markl's methods, has prepared plague toxins with the same degree of certainty as broth toxins of the Shiga dysentery bacillus. A medium consisting of ordinary peptone broth, with or without the addition of 1 per cent. normal horse serum, is distributed into flasks in such a manner as to ensure good access of the culture to air. The initial reaction of the medium is slightly alkaline (pH 7·0 to 7·3). The cultures are kept at room temperature (10 to 15° C.) for about two months. At the end of this time toluol is added to each flask; the flasks are put aside for a few days to allow the toluol to act; and the cultures are then freed from bacilli by filtration through a Seitz filter.

Extraction methods.

The method of Lustig and Galeotti. Lustig and Galeotti (1897) were the first workers to succeed in extracting a specific antigen from plague cultures by chemical methods. They treated a bacillary suspension with 1 per cent. solution of potassium hydrate, and two hours later just over-neutralized the mixture with 0·5 per cent. acetic acid, until white flocculi began to appear. The flocculent precipitate proved to be a toxic nucleoprotein and to possess immunizing properties.

The method of Sydney Rowland (1910). In the work he carried out on behalf of the Advisory Committee on Plague in India, Rowland obtained a toxic nucleoprotein by treating with anhydrous sodium sulphate the bacillary paste scraped from surface growths on agar. The cultures were first killed by means of chloroform, and the washed spun paste of bacilli was thoroughly mixed in a mortar with the anhydrous salt until a dry powder was obtained, which was then dissolved in twice its weight of warm distilled water; in this way a solution of sodium sulphate was obtained of such a strength as to precipitate completely all the bacterial proteins. The solution was filtered, and the residue of bacteria was suspended in distilled water, whereupon solution of the nucleoprotein took place. When examined by dark-ground illumination after the sulphation process, the bacilli looked normal, but were very fragile and stained with difficulty. Rowland thought that the treatment with sodium sulphate modified the consistence and permeability of the bacterial envelope.

Other methods.

Numerous methods have been tried with the object of preparing an effective antigen from the plague bacillus, either as a vaccine for human use or for experimental purposes. To ensure sterility of the product the bacilli have been killed by heat or desiccation, or by treatment with glycerin or alcohol. Purification and concentration of the specific substances have been attempted by adding ammonium sulphate, sodium sulphate, or absolute alcohol as precipitants to filtered broth cultures or autolysates. Exudates from infected guinea-pigs, and the bubo and organs of animals dead of acute plague have been proposed as the source of a vaccine. Although all such preparations are likely to contain immunizing substances, none have gained widespread acceptance in the practice of vaccination against human plague, because they are too elaborate for production on a large scale. Research is still needed to discover a plague vaccine which will give the maximum degree of immunity with the minimum amount of discomfort to the recipient; to this end a simple process which will ensure a safe and sterile product is an important desideratum.

A brief reference may be made to the work of Eberson (1917[1 & 2], 1918) on the 'proteotoxins' of the plague bacillus. They are liberated when the sensitized bacilli are digested in fresh guinea-pig serum for about 14 hours at 37° C. The resulting material, when injected intravenously into experimental animals, rapidly causes fatal shock-like symptoms. These poisons are apparently related to the anaphylatoxins of Friedberger. Eberson speculates on the role played by them in the mechanism of infection, but since the interpretation of his experiments is dubious, it is best to await the results of further work on the subject.

Strong (1906, 1907) has recommended living avirulent cultures as a vaccine for human use, and has suggested suitable controls and precautions to secure the safety of the procedure. Our knowledge of the conditions which influence the degree of virulence of plague cultures is still incomplete, and for this reason vaccines composed of the living culture have not yet been adopted by plague administrators.

General Characters of the Endotoxin and the Immunizing Substance.

The chemical reactions of the toxic nucleoprotein extracted from the plague bacillus by Rowland's method.

The product extracted from plague growths by Rowland's sulphate method gives the reactions of a nucleoprotein, including the biuret, xanthoproteic, glyoxylic, Molisch's, and Millon's reactions. When acid is added to the solution a precipitate is thrown down, which is soluble in dilute alkali. The precipitation limits of the protein by sodium sulphate correspond to the densities 1,140 to 1,200. The immunizing substance is closely associated with the protein; attempts made by Rowland to effect a separation by filtration through Martin's gelatin filter did not succeed. The nucleoprotein constitutes about one-tenth by weight of the whole bacillus; an average agar slope (10,000 million bacilli) yields 1 mgm. of nucleoprotein.

The relation between the virulence of strains of the plague bacillus and the toxicity of extracts derived from them.

The observations of Rowland indicate that the virulence and the toxicity of plague cultures are not related. Thus the toxicity for white rats of a solution of nucleoprotein prepared from plague bacilli grown on inactivated horse-serum agar, a medium which heightens the virulence of the plague bacillus, was about the same as that of a preparation from a less virulent broth-agar strain. Again, the toxicity for white rats of a nucleoprotein solution prepared from an avirulent culture was but slightly lower than that from a virulent strain grown under the same conditions. A completely avirulent strain, isolated 27 years ago from a case of pneumonic plague in Vienna, yielded a toxin with an m.l.d. for a mouse, when given intravenously, of 1/75 c.cm.; the average m.l.d. of 25 other toxins prepared at the same time in the same way from recently isolated cultures was 1/100 c.cm. (Petrie, unpublished work).

Factors that influence the stability of the endotoxin and the immunizing substance.

Temperature. The observations on the influence of temperature are conflicting, and the discrepancies are perhaps due to unrecognized differences in the experimental conditions, and particularly in the pH of the solutions containing the toxin or the immunizing substance. The gradual loss of toxicity from storage at ordinary laboratory temperatures, and the loss which follows exposure for short periods (½ to 1 hour) at temperatures within the limits 37 to 70° C., probably involve the same physico-chemical changes. Thus a comparatively low temperature acting for a long period has the same effect as a high temperature acting for a short period. Plague toxins in fluid form are not very stable when stored at room temperature: at 37° C. they suffer a considerable loss of toxicity. The workers in the Haffkine Institute, Bombay (Morison, Naidu and Avari, 1924[1, 2 & 3]), have frequent opportunities for noting the toxic action of Haffkine's prophylactic fluid on susceptible *M. rattus* (Madras rats). Although the cultures used for its preparation are heated to an average temperature of 55° C. for 15 minutes, 35 per cent. of these rats die of toxæmia after a dose of 0·5 c.cm. of the vaccine, and in some the post-mortem signs resemble so closely those of a typical naturally infected rat that it is impossible to distinguish the lesions by the naked eye. As is well known, Haffkine's vaccine occasionally gives rise to unpleasant toxic symptoms, both local and general, in man. After exposure to 60° C. for half an hour a toxic effect is still demonstrable in susceptible animals, but this temperature appears to be near the limit of tolerance for the 'lethal' toxin. A temperature of 100° C. for even a brief time completely destroys the toxin. The evidence upon the effect of heat on the toxicity of bacillary suspensions shows that these react in much the same way as the filtered toxin. The experience of the present writer leads him to believe that plague toxins, whether prepared from broth cultures or by extraction methods, retain their toxicity well if kept strictly at a constant low temperature, not higher than 3° C.

When a broth toxin has been heated within the limits 50 to 70° C. for 1 hour, it is still capable of functioning as an immunizing agent, although the toxicity is greatly reduced (Markl, 1898, 1901 ; Kossel and Overbeck, 1902 ; G. Dean, 1906) ; exposure to 100° C. destroys the antigenic substance. Bacillary suspensions after being heated to 65° C. for 1 hour are still antigenic (The German Commission).

The nature of the degradation process associated with the weakening of the lethal action of plague toxin is not yet understood, but Rowland's observations (1911) on the presence of a proteolytic enzyme in the bacterial protein are perhaps significant. He found evidence of proteolysis in bacillary suspensions and in solutions of the nucleoprotein, when incubated at 37° C. under toluol. The progressive hydrolysis of the protein is accompanied by a marked reduction of toxicity ; whereas the immunizing value decreases to a slight extent only, even after the lapse of one month. The graph in Rowland's article illustrating this relation is reminiscent of graphs that show the relation of the toxicity of diphtheria toxin to its combining power as measured by the L+ dose, and it is very likely that, as with diphtheria toxin, the explanation lies in the partial transformation of the lethal toxin into a toxoid modification. Stevenson and Kapadia (1924–5) ascertained that storage of Haffkine's vaccine at the room temperature of Bombay (23 to 29·5° C.) gradually diminished its toxicity for rats while affecting to a much less extent the immunizing value. In experiments carried out with the purpose of testing antitoxic sera against various test toxins, MacConkey (1912) obtained indications that the toxin in an old autolysed solution of plague nucleoprotein had undergone a partial change into toxoid. This idea is supported by the fact, also noted by MacConkey, that such a comparatively atoxic substance actively stimulates the production of plague antitoxin in the horse.

The general conclusions regarding the influence of temperature upon the endotoxin and the immunizing substance may be stated as follows : (1) Both are thermolabile, but of the two the immunizing substance withstands heat the better ; (2) their deterioration is a function of time as well as of temperature ; (3) under unfavourable conditions of storage the lethal toxin is progressively changed into an atoxic substance (toxoid), which, however, possesses antigenic value.

The influence of preservatives upon the toxin and the immunizing substance. Toluol is the best preservative for plague toxin. Chloroform, too, is suitable, but according to Rowland it forms with the toxic nucleoprotein a loose reversible combination which is less toxic than the original solution. Thymol was found by Rowland to lessen both the toxicity and the vaccinating value of a bacillary suspension. Phenol in a concentration higher than 0·5 to 0·75 per cent. has a harmful effect on the antigen. From the work of Morison, Naidu and Avari (1924[2]) it seems that in bacillary suspensions that are treated by the formalin-acetone method of Dreyer, or by 1 per cent. phenol, the immunizing substance is injured to a greater extent than when the cultures are heated to an average temperature of 55° C. for 15 minutes, and then phenolized to a concentration of 0·5 per cent. Bacilli treated by the formalin-acetone method were not toxic when tested in considerable doses on rats, and they were incapable of producing immunity to the living virulent culture.

The susceptibility of experimental animals to the toxin.

The mouse and the rat are susceptible to the toxin, and are, therefore, suitable for testing purposes; the guinea-pig and the rabbit show considerable resistance. The action of the toxin on mice is a rapid one; whether administered subcutaneously or peritoneally, it causes death as a rule within 18 hours. The present writer (unpublished work) has prepared broth toxins from a large number of cultures isolated in many countries and of varying ages since the date of isolation, and has found that the toxicity may be conveniently measured by injecting graduated doses intravenously into mice. In one batch of 53 toxins, the m.l.d. ranged from 0·1 to 0·0033 c.cm. with an average m.l.d. of 0·013 c.cm. (1/76 c.cm.); in another batch of 26 toxins the m.l.d. ranged from 0·1 to 0·0025 c.cm. with an average m.l.d. of 0·0095 c.cm. (1/105 c.cm.). Comparative figures for the m.l.d's. of 17 broth toxins prepared from the Shiga-dysentery bacillus are: 0·04 c.cm.; 0·002 c.cm. and 0·0053 c.cm. (1/190 c.cm.) (Petrie, 1927). Mice show *post mortem*, few, if any, naked-eye signs of plague toxæmia, partly, no doubt, because death occurs so quickly.

White rats die usually after 48 hours when the toxin has been given subcutaneously, and after 24 hours when given intraperitoneally. The lethal dose of a broth toxin is from 0·05 to 0·5 c.cm. per 100 gms.; a smaller amount kills by the intraperitoneal route than by the subcutaneous route. The nucleoprotein prepared by Rowland killed rats in a dose of 0·1 mgm. Rats that recover from a sublethal dose or from a toxin-antitoxin mixture (MacConkey, 1912) may die later with marasmus similar to that seen in human plague. The post-mortem signs in white rats resemble those seen in naturally infected wild rats; the subcutaneous congestion is often well-marked, and hæmorrhages in the organs, the characteristic granular condition in the liver and a clear pleural effusion may be observed (personal experience).

Guinea-pigs and rabbits withstand large doses of the toxin; 27 mgm. of plague nucleoprotein—a quantity 270 times the m.l.d. for white rats—did not kill guinea-pigs (Rowland). Nevertheless, a dose of the toxin incapable of causing acute symptoms in the guinea-pig may induce a condition of marasmus some weeks later. Goats and horses are susceptible to the toxin, and if given an excessive dose may die from a toxæmia.

Analysis of the Antigenic Constitution of the Plague Bacillus.

The plague bacillus appears to be built up of two varieties of protein, one soluble in distilled water and saline and containing the toxic and the immunizing substances, the other insoluble in distilled water and saline and possessing no toxic and immunizing properties (Rowland; Morison, Naidu and Avari, 1924[1]). When the soluble protein is extracted the bacillus retains its shape, but is fragile and readily breaks up (Rowland, 1910). The inference may be drawn that the cell-membrane is composed of the insoluble fraction.

There is conclusive evidence for the existence of a specific toxin, which is associated with the soluble protein of the bacillus, and which is set free in broth cultures by maceration of the bacilli after their death or in bacillary suspensions by extraction methods. The action of the toxin on susceptible animals, and its neutralization with antitoxin, characterize it as a definite specific antigen. As will soon appear, the soluble protein, when inoculated into a susceptible animal, gives rise to an opsonizing substance which, in a subsequent experimental infection, facilitates the ingestion of bacilli by the leucocytes, and which thus takes an important part in the mechanism of defence.

Although, in this account, the term immunizing substance has been used in a general sense there is no clear proof of the existence of an independent antigen, apart from the toxin and the opsonic antigen, which confers an active immunity to the living virulent culture. Here it is important to note that the preparations used by various workers in the numerous experiments on active immunization have all contained toxin, toxoid, or a mixture of these antigens.

The toxic and immunizing properties of the nucleoprotein are without doubt closely allied: Rowland believed that for a vaccine to be efficient it must originally have had a toxic action. He pointed out that the plague nucleoprotein is toxic for rats, and, when given in suitable sublethal doses, will protect them against the living culture, but that it is not toxic for guinea-pigs and is unable to protect them against an infection. The toxic and immunizing effects cannot be dissociated by the aid of heat, because both are abolished at about the same temperature. The immunizing substance certainly does not belong to the group of thermo-stable antigens, which can withstand a temperature of 100° C. The rapid induction of the immunity conferred by the nucleoprotein seems to bear a relation to the characteristically rapid absorption of plague toxin or to a rapid opsonic response, and is less consistent with a more slowly acting bacterial antigen of the thermo-stable type. The discovery of a method of testing which will make it possible to search for and measure small amounts of plague antitoxin in the blood of vaccinated animals may help to solve the problem of the nature of the immunizing substance.

The later work of Rowland tended towards the conception of antigenic efficiency as a quality which varied according to such factors as the source of the culture, its virulence, and the influence of the composition of the medium upon the virulence of the culture. A review of his experiments on prophylaxis and of the work of others suggests that three chief factors control the results: (1) the thoroughness of the immunization, (2) the virulence of the test culture, and (3) the size of the test dose. Due account should be taken of these variables before drawing conclusions upon the antigenic efficiency of vaccine preparations.

The cross-agglutination experiments of Otto and the experiments of Hetsch and Rimpau (Kolle, Hetsch and Otto, 1904) on the production of a curative antibody from various strains, strongly favour the view of a simple rather than of a complex and variable antigenic constitution of the plague bacillus.

Finally there is some evidence (Markl and G. Dean) that an antigen exists in the plague bacillus which, in the course of immunization by the intravenous method, gives rise to an antibody in the serum capable of curing infection in experimental animals, although the serum is feebly or not at all antitoxic. Whether this antigen is of the thermo-stable type or not is as yet unknown.

THE PRODUCTION OF ACTIVE IMMUNITY TO THE TOXIN AND TO THE LIVING VIRULENT CULTURE.

Markl, beginning with sublethal doses of a broth toxin, immunized mice against the toxin; they withstood 100 lethal doses. Rowland in a similar way immunized white rats; the larger the immunizing dose the more resistance they showed to the test dose of toxin. The evidence is conclusive that active immunity to the living virulent culture is best achieved by vaccination with living cultures (The German Commission, Kolle and Otto, 1903[1 & 2]; Strong, 1906–7). Kolle and Otto carried out experiments with an old culture whose virulence had been weakened in some unknown manner by Maassen. Guinea-pigs tolerated a dose of one agar-slope culture, when given subcutaneously or intraperitoneally; no pathogenic action was noted in rats and mice. Sixty-four per cent. of guinea-pigs, 72 per cent. of rats, and 60 per cent. of mice were protected by its means; the corresponding death-rates resulting from the lethal effect of the immunizing culture were 22, 2·3 and 18 per cent. Strong (1907) protected 26 per cent. out of 15 guinea-pigs by means of a killed agar-grown vaccine; when a living avirulent plague vaccine was used 72 per cent. of 64 animals survived the test-dose; on another occasion 88 per cent. of 41 guinea-pigs were saved (cited by Schütze, 1927).

The work of many observers has shown that a definite degree of immunity can be conferred upon rats and guinea-pigs by means of various preparations derived from plague cultures. Rowland; Morison, Naidu and Avari; Schütze; and Burgess (1927) are recent contributors to this aspect of the subject. On account of the difficulty of standardizing the experimental conditions the results of different workers are not comparable. As already stated, the thoroughness of the preliminary immunizing procedure is important, and thus multiple injections are better than a single injection ; moreover, the virulence of the test culture and the amount given as a test dose greatly influence the survival rate: in the recorded experiments from 0 to 50 per cent. of the control animals survived. When a considerable dose of the prophylactic is given, a number of the test animals die from toxæmia, and the survival rate of the remaining animals never reaches 100 per cent. As an example, some of the results obtained by Morison, Naidu and Avari (1924[1]) may be cited. Haffkine's vaccine, when given in a dose of 0·5 c.cm. to susceptible (Madras) rats, killed 35 per cent. from toxæmia; among the remaining animals an immunity was produced which gave a survival rate after a test dose of living culture of 44 per cent. as compared with 5 per cent. in a control group. The supernatant fluid of the vaccine gave like figures—a result which shows incidentally that the soluble bacterial protein is the active constituent of Haffkine's vaccine.

Schütze (1925) compared the immunizing efficiency of three vaccine preparations : (*a*) the Lister Institute vaccine, a phenol-killed serum-agar vaccine (see p. 154), (*b*) the Lister vaccine made from a stock non-body strain, (*c*) an agar-grown heat-killed (60° C. for 1 hour) vaccine—all prepared from the same strain, and (*d*) Haffkine's prophylactic fluid. When tested on guinea-pigs the first-mentioned vaccine proved to be the most efficacious, whereas in experiments with rats the vaccines immunized equally well.

The conclusion may be drawn that plague vaccines confer a definite immunity upon the rat and the guinea-pig ; that the rat is more easily rendered immune than the guinea-pig ; and that the degree of protection conferred depends on the thoroughness of the immunization and the severity of the testing procedure. The test conditions arranged in most of the recorded experiments are probably more severe than those of the natural mode of infection.

The Onset and Duration of the Immunity to the Living Culture conferred by the Antigens of the Plague Bacillus.

Rowland (1912) found that in the white rat immunity to the living culture was distinct 24 hours after vaccination with his nucleoprotein solution, and that it reached a maximum on the third day; it was unimpaired at the end of three months and was still appreciable at the end of five months. Stevenson and Kapadia (1924–5) showed that the immunity conferred on rats by a prophylactic dose of Haffkine's vaccine began within 24 hours of the inoculation. The experiments of the German Commission on monkeys indicated a more gradual development of the immunity with a maximum response on the seventh day. The differences in the rate of response in the white rat and the monkey are consistent with Brooks's (1914) observations on the development of opsonins ; the maximum response in the white rat occurred on the first or second day, and in man on the fifth or sixth day.

Experimental Work on the Various Methods of Preparing and Administering Plague Vaccines.

Naidu and Jang (1926[1]) tested the immunizing efficiency of vaccines that were sensitized with rabbit, sheep and horse immune sera, and concluded that, when given to susceptible (Madras) rats, they had no advantage over the unsensitized vaccines. Pirie found sensitized vaccines of little use in the prophylaxis and treatment of plague in gerbilles. Cuti-vaccination with heat-killed vaccines has been tried on the guinea-pig by Girard and Quimaud (1924), but without success ; and like experiments by Pirie with formalin-killed vaccines on South African rodents, were also unsuccessful. Leger and Baury (1923[2]) experimented on guinea-pigs, rabbits and monkeys, and thought they obtained an immunizing action with plague vaccine when given by the mouth after administration of ox-bile according to Besredka's procedure. Morison, Naidu and Avari (1924[3]) repeated this work, but failed to confirm it. They regard rats as unsuitable for the oral administration of bile and vaccine, and were unable to confer immunity upon guinea-pigs by this method.

The Mechanism of Active Immunity to Plague.

The observations of Markl (1903) and of Rowland (1912) on the events that take place during the course of peritoneal infection in the white rat show that lysis and phagocytosis of the bacilli compete with the rate of multiplication of those which remain free in the exudate. On the one hand, the efficiency of the phagocytic and lytic processes is inversely related to the virulence of the infecting strain ; on the other hand, the rate of multiplication depends upon the virulence of the infecting strain and upon the immunity level of the tissues of the host. Thus a virulent culture multiplies with difficulty in an immune rat, just as an avirulent culture has little power of multiplication in the tissues of even a susceptible animal. Markl (1903) found that the administration of a prophylactic or simultaneous dose of immune serum to a rat markedly promoted phagocytosis and lysis of virulent bacilli, and that sensitized virulent bacilli, when introduced into the peritoneal cavity of the rat, were readily phagocytosed.

The phagocytic part of the mechanism is readily explained on the basis of an opsonic action. Douglas (cited by Brooks, 1914) found a good opsonic response in man after inoculation with Haffkine's prophylactic fluid. Brooks (1914) obtained an opsonic response in white rats by injecting them with the soluble bacterial protein; the insoluble portion left after extraction was without effect. In rats, in the early stages, the opsonic response and the degree of protection developed together. Body-strains, that is, strains cultivated direct from an infected rat, were insusceptible to phagocytosis, whereas laboratory strains were readily taken up by the leucocytes.

The bacteriolytic part of the defence mechanism is not easily explained, since most workers have failed to demonstrate a bactericidal element in immune serum, although it contains specific amboceptors.

THE PRODUCTION OF PASSIVE IMMUNITY TO THE TOXIN AND TO THE LIVING VIRULENT CULTURE.

The Production of Plague Antitoxin.

Markl immunized animals against plague toxin and was the first to demonstrate a specific antitoxin in their serum. G. Dean, Rowland, and MacConkey, working in the serum-laboratories of the Lister Institute, prepared an antitoxic serum from horses. Dean obtained a serum of which 0·02 c.cm. neutralized 20 rat-m.l.d's. (1 c.cm. = 1,000 rat-m.l.d's.). In 1909, Rowland immunized a horse with the bacterial protein prepared by means of his sodium sulphate method. When tested on rats, 0·1 c.cm. of the serum neutralized 7·5 rat-m.l.d's. of the toxic nucleoprotein (1 c.cm. = 75 rat-m.l.d's.). The serum protected white rats, when given the day before the administration of a test-dose of a culture of moderate virulence. MacConkey (1912) carried out further work with 'toxins' prepared by Rowland, and particularly with reference to the titration of the antitoxin. The neutralizing power of the sera obtained by MacConkey was equivalent to 300 to 400 rat-m.l.d's. per c.cm.; with one sample a value of 750 rat-m.l.d's. per c.cm. was reached. He showed that the antitoxin is associated with the pseudoglobulin fraction of the serum, and can be concentrated by the usual methods. The antitoxic sera prepared by Rowland and MacConkey were tested in Bombay by the Plague Research Commission (1912). There was a difference of only about 10 per cent. in favour of the serum-treated cases—a result which was regarded as of doubtful significance. In the experience of the present writer plague antitoxin in high concentration, for reasons that are not obvious, is not easily produced in horses.

There are difficulties in the titration of the antitoxin that have not yet been overcome. Essential desiderata are the discovery of a method of preparing sufficiently powerful and stable test toxins, the introduction of a standard serum, and the adoption by its means of an arbitrary unit of antitoxin. It is probable that the strength of plague toxins is measured only in a crude fashion in terms of the m.l.d., and that toxoids in varying proportions exist not only in different toxins but in any one toxin according to its age and the conditions under which it is kept. If this view is correct, there will be no progress in the titration of plague sera until the test dose of toxin is standardized with the aid of a standard serum.

The Conferment on Experimental Animals of Passive Immunity to the Living Virulent Culture.

A serum intended for use in human plague should certainly contain an antitoxin for the endotoxin of the bacillus. The cellular damage is caused to a large extent by the action of the toxin, so that the speedy neutralization of free toxin in the tissues is an important therapeutic aim. The conclusion cannot, however, be drawn that a purely antitoxic serum is sufficient. Evidence already adduced makes it clear that it is necessary also to inhibit the multiplication of the bacilli in the bubo or other infected tissues in order to check invasion of hitherto uninfected tissues, and at the same time to put an end to the liberation and absorption of toxin. A completely effective therapeutic serum for plague must, therefore, contain in adequate amounts an antibacterial as well as an antitoxic element. Markl and G. Dean prepared sera which were feebly or not at all antitoxic, but which proved to be curative, when given to experimental animals. As already mentioned, such sera initiate a clearing process by virtue of their phagocytic and lytic properties, and also inhibit the multiplication of the bacilli in the tissues of the host.

The results of prophylactic and curative experiments with antiplague sera, which have been recorded by various observers, cannot be said to be notably successful, although they indicate that the serum has some protective action. In considering the significance of these experiments the fact should be recognized that even now there exists no certain and accepted method of titrating the strength of the antitoxic and antibacterial components of plague sera. Moreover, the results of experiments with plague sera depend greatly, as in the case of vaccines, upon the virulence of the test culture, the size of the test-dose, and the mode of its inoculation. As an example, the experiments of Kolle and Martini (Dieudonné and Otto, 1927) may be cited. They tested antiplague serum obtained from the Pasteur Institute, Paris, on 500 experimental animals, mostly rats and guinea-pigs. It is probable that, when the experiments were carried out, this serum was antibacterial but only slightly antitoxic. The summed results are shown in Table III, and the following details may be added: A prophylactic dose saved about 50 per cent. of the rats, but the curative action was very slight. Of 16 guinea-pigs that were given a prophylactic dose, 9 died ; a curative action was not apparent except after a cutaneous infection with weakly virulent cultures, when about 50 per cent. were saved. In experimental plague pneumonia, the serum had no prophylactic or curative action in mice and guinea-pigs, but 2 c.cm. protected rats with certainty when given from 1 to 20 hours before the infecting dose.

TABLE III.

Summarizing the results obtained by Kolle and Martini (Dieudonné and Otto, 1927) with antiplague serum when tested on experimental animals.

	Plague serum.	Normal serum.	Controls.
Survivors of the whole series of experiments	34 per cent.	7 per cent.	0
Survivors when a prophylactic dose was given	13·1 „	5·7 „	0
Survivors when a curative dose followed inoculation with a virulent culture	8·9 „	4·5 „	0

The Transmission of Plague by the Rat-Flea.

AN ACCOUNT OF THE WORK OF THE PLAGUE RESEARCH COMMISSION IN INDIA (1906, 1907, 1910, 1912).

The Experimental Transmission of Plague by the Rat-Flea.

During the first two years (1905–7) of their work in Bombay, the Commission brought forward much evidence for the view that the rat-flea plays an important role in the spread of plague. They began by repeating the transmission experiments of Gauthier and Raybaud (1903), and adopted the type of cage devised by them. The cage was divided into two compartments, one of which contained a healthy, and the other an infected rat ; the partition which separated the animals allowed of a free exchange of rat-fleas, but prevented contact of the healthy rat with the body or excreta of its infected neighbour. Experiments were made under these conditions with 16 tame white rats and with 50 wild Bombay rats, *M. rattus* and *M. norvegicus*, as the receptive animals, with the result that 30 of the total of 66 (45 per cent.) contracted plague. There was experimental evidence that at this time about 40 per cent. of the wild rats in Bombay remained healthy when inoculated cutaneously with small doses of a plague culture, so that the chances of successful transmission by fleas were correspondingly diminished. To exclude any possibility of aerial infection, in the next series of experiments fleas from rats dying of plague were transferred to a tame rat or to a wild Bombay rat in a flea-proof cage ; 21 animals out of 38 (55 per cent.) contracted plague. The rat-fleas used in the experiments were those which infested the rats in Bombay ; all of them were regarded by the Commission as being *X. cheopis* Rothsch. ; it is now known that probably some were *X. astia*, a species of rat-flea which was described in 1914 by Rothschild.

The Experimental Production of Plague Epidemics.

The object of these experiments was to discover the importance of the rat-flea in the production and spread of epidemic plague. Some workers held the belief that contact with the excreta of plague-infected rats or the eating of food contaminated by their excreta often promoted the spread of the disease among rats and human beings. The experiments were carried out in a block of huts or godowns built for the purpose by the late Surgeon-General Bannerman, I.M.S., and Lt.-Colonel W. Glen Liston, C.I.E., I.M.S., before the Commission began its work. The supply of fleas was at first a natural and fortuitous one from wild rats living in or visiting the roofs, but new godowns were afterwards built which were planned to be strictly rat-proof and flea-proof ; and yet conditions in Bombay were so favourable for the breeding of rat-fleas in buildings that precautions had to be taken to keep the control godowns as free as possible from fleas. The experimental animal was in the first instance the guinea-pig, but later wild Bombay rats were used in order to meet the criticism that the Commission had not taken into account the cannibalistic habits of wild rats, which were assumed to further the spread of the epizootic.

Experiments with guinea-pigs.

These experiments showed that epidemic plague did not occur when healthy guinea-pigs lived in intimate contact with plague-infected guinea-pigs under conditions that prevented the access of fleas. On the other hand, under similar conditions, plague spread among the healthy animals in the experimental huts in which fleas were abundantly present. In one experiment fleas from guinea-pigs that were dying from plague were transferred to a ‘ clean ’ godown which contained 49 healthy guinea-pigs : an epidemic was thus started and was maintained by the frequent introduction of fresh lots of fleas until every guinea-pig had died. When an epidemic had destroyed a community of guinea-pigs the contagion still remained in the godown, and was effective in proportion to its infestation with fleas.

The accessibility of the exposed animals to fleas was varied by (1) allowing some to run loose over the floors ; (2) placing some in cages accessible to fleas and two inches from the ground; and (3) placing some in cages inaccessible to fleas because they were hung two feet from the ground. This type of experiment was repeated on several occasions, with the result that the infestation of the guinea-pigs with fleas and the rate of mortality from plague were in the order as given above : the guinea-pigs placed two feet from the ground were fully protected from the infection.

Experiments with and without fleas were carried out simultaneously. In 6 contact experiments at different seasons of the year, in godowns that were free from fleas, not one guinea-pig died of plague. In 3 of these experiments, the contact with infected animals was kept up by the daily addition of flea-free plague-infected guinea-pigs for a period of from 14 to 21 days, and still none contracted the disease. The godowns were never cleaned out, and therefore, there were unrestricted opportunities for contact with the excreta of infected guinea-pigs and for eating food contaminated with their excreta. The Commission inferred that ‘ contact ’ in the absence of fleas did not give rise to an epidemic, and that in the flea-infested godowns fleas alone were responsible for the epidemics.

While the rat epizootic was increasing in Bombay City, the guinea-pig epidemics ran a rapid course, whereas, during the non-epidemic season in Bombay the epidemics in the godowns were more or less protracted and were incomplete, for a few of the guinea-pigs escaped the infection. Experiments that were made at the same time of the year and under similar conditions showed that the rate of progress of a guinea-pig epidemic in a slightly flea-infested godown differed from that in a godown which possessed an abundance of fleas ; the fewer the fleas the slower was the rate of progress. In a slightly flea-infested godown the epidemic took 68 days to kill 25 guinea-pigs, but in a heavily flea-infested godown, 24 of 25 guinea-pigs died in 6 days.

Twenty-two monkeys were put into the infected godowns, each in a special cage. Ten monkeys were kept in cages furnished with fine metallic gauze as a protection from attack by fleas: all lived. Five of the 12 unprotected monkeys died.

Experiments with wild Bombay rats (*M. rattus*).

In a fresh series of experiments the Commission substituted in place of guinea-pigs wild Bombay rats (*M. rattus*), which had previously been carefully freed from fleas. Six new godowns built of reinforced concrete were used; 3 were stocked with rat-fleas each week, and 3 were kept flea-free as controls.

TABLE IV.

Comparing the plague mortality among rats (Bombay *M. rattus*) in control godowns that were virtually free from fleas and in flea-infested godowns.

Serial No. of Experiment.	Date of Experiment (1909–10).	Control Godowns (almost flea-free).			Flea-infested Godowns.			
		No. of rats exposed to risk.	No. of rats that died.	No. of fleas found in godowns.	No. of rats exposed to risk.	No. of rats that died.	Percentage mortality.	Average No. of fleas per rat.
1	24 Sep.–2 Nov.	45	1	1	45	5	11	4
2	5 Nov.–13 Dec.	45	0	2	45	17	38	23
3	21 Dec.–24 Jan.	45	0	some	45	27	60	37
4	3 Feb.–10 Mar.	45	0	3	45	19	42	16
5	22 Mar.–2 May	45	0	0	45	28	62	21
6	10 May–13 June	45	0	0	45	30	67	14
7	25 June–1 Aug.	45	0	2	45	24	53	22
8	15 Aug.–26 Sep.	45	0	0	45	16	36	16
		360	1		360	166	46	
		0·3	per ce	nt.				

Table IV summarizes the results of 8 experimental epidemics from September, 1909, to September, 1910. A rigorous technique was adopted for clearing the rats and godowns from fleas. The experiments justify the conclusion that infection spreads readily from rats that have been inoculated with plague to healthy rats living with them, if a considerable number of rat-fleas are present; and that under similar conditions, except that fleas are absent, or present only in very small numbers, no epidemic will occur. No evidence emerged that alimentary infection was concerned in the production of the epidemics. The table shows a fair relation between the degree of flea infestation of the godowns and the percentage of rats dying from plague, except in Experiments 5 and 6: the explanation probably is that, in the rats which were inoculated with a plague culture in order to start the epidemic, the degree of septicæmia was greater in

Experiments 5 and 6 than in any of the others, and so a larger proportion of the fleas were infected. Of the rats used in these experiments, a number varying from 30 to 70 per cent. according to the test-dose, were immune when inoculated subcutaneously, and therefore a 100 per cent. mortality from flea transmission could not be expected.

An experimental epidemic in an infected village near Bombay City.

This village, Sion Kolivada, situated a few miles to the north of Bombay City, was chosen by the Commission for observation, when free from plague, with the object of discovering the mode of introduction of plague into it in the event of its becoming infected. Plague broke out here in due course, and the probable cause was the importation from Bombay of infected rat-fleas which started an epizootic among the house-rats. After one or two cases of human plague had occurred the inhabitants of the village left it voluntarily, and occupied a rude camp constructed by themselves in the neighbourhood.

The Commission then placed guinea-pigs in the houses, a procedure which proved to be ideal for studying the relation of the epizootic to the infectivity of the houses. A severe epidemic spread among the guinea-pigs under conditions which altogether excluded the conveyance of the infection from one guinea-pig to another. This experiment revealed the normal course of an epidemic uncomplicated by circumstances that, in human epidemics, tend to scatter infection in a haphazard fashion through human agency; the regular centrifugal spread of plague among the guinea-pigs was due solely to the dispersal of infection by the excursions of rats. The observations in the village indicated that the movements of rats are usually limited to short excursions between adjoining houses, and this view receives support from an experiment with marked rats made in a village in Upper Egypt (Petrie and Todd, 1923).

Forty-five per cent. of the buildings in Sion village were infected; all the rats infesting the houses were *M. rattus*. The rate of diffusion of the rat epizootic was slow; it took 6 weeks to travel about 300 feet. As is usual when demolition methods cannot be employed, the number of plague rats that were found was small. The infection persisted among them for two months. The Commission concluded from their observations that, corresponding both in time and in place with the epizootic, there was a dispersal of an infecting agent within the houses, and that this agent was the infective rat-flea.

Experiments in plague houses.

The Commission extended their experiments to plague-infected houses in Bombay. Eleven thousand plague deaths were reported from the City and Island during the twelve months ending 30th September, 1906, so that there were ample opportunities for investigating the problem of the infectivity of houses. Guinea-pigs that were allowed to run free in plague houses often attracted many fleas, most of them rat-fleas; as a result 29 per cent. of these guinea-pigs died of plague. The Commission examined in the same way a group of houses that had been recently disinfected by thoroughly washing the floors and walls with an acid solution of perchloride of mercury of a strength of 1 in 750 ; a few had been fumigated with sulphur. Numerous fleas were caught on guinea-pigs set free in these disinfected houses, and 29 per cent. of the guinea-pigs died of plague: a result indicating that disinfection is useless in reducing the infectivity of plague houses. Fleas transferred from plague-infected rats that were found dead or dying in houses conveyed plague to healthy animals in flea-proof cages in the laboratory. Moreover, fleas transferred from guinea-pigs that had been kept for a few hours in plague houses infected fresh animals in the laboratory.

Monkeys, guinea-pigs, and white rats, confined in specially designed cages, were placed in plague houses in pairs. The cages were so contrived that both animals were protected from soil and contact infection and both were equally exposed to aerial infection, but whereas the one was protected from fleas the other was not so protected. In some of the experiments the mode of protection was a metallic curtain of a mesh fine enough to prevent fleas passing through it, and in others a layer of ' Tanglefoot ' which was broad enough to prevent fleas hopping across it to reach the test animal. None of the animals in the cages to which fleas were denied access contracted plague, whereas several of the animals in cages that were open to invasion by rat-fleas died of the infection. Many fleas were caught on the ' Tanglefoot ', and in the stomach of some of them abundant bacilli microscopically identical with *B. pestis* were found. Of 85 human fleas that were dissected only 1 contained plague-like bacilli, but of 77 rat-fleas 23 were thus infected. During the epidemic of 1907 further experiments of this nature were carried out with similar results.

In the Commission's Report a table will be found which summarizes the flea census in plague houses in the epidemic of 1906, and which shows that the average number of fleas per house in the group ' definitely proved to be plague-infected ' was three times the average per house in the group ' presumably plague-infected '. A similar table for the epidemic of 1907 classifies the houses as (1) ' proved to be infected ', (2) ' presumably infected ', and (3) ' not infected ' ; the corresponding averages of fleas per house are 29, 10·3 and 2·5. Plague-like bacilli were found in the stomach of 41 out of 130 fleas (31·5 per cent.) that were caught on guinea-pigs in plague-infected houses.

Confirmatory experiments were made by the Commission in the Punjab villages, Kasel and Dhand.

Evidence derived from the Regional Distribution of the Primary Bubo in Confirmation of the View that the Rats and Guinea-pigs which died of Plague in the Foregoing Experiments received their Infection through the Agency of the Rat-Flea.

The Commission fed infected rat-fleas at different sites upon guinea-pigs, and in nearly every instance the position of the bubo was related to the site of the meal; this observation shows that the primary bubo takes its origin in the group of glands that is in direct lymphatic connection with the area of skin through which the plague bacillus enters the tissues of its host. If, then, a characteristic distribution of the primary bubo is found in a group of rats or guinea-pigs that have been experimentally infected by rat-fleas, it is reasonable to conclude that a similar distribution in any other group indicates that transmission by rat-fleas has been a predominant factor. Tables V and VI furnish convincing evidence that the test animals, both rats and guinea-pigs, which died of plague in the godown epidemics, in Sion village, and in plague houses in Bombay, were infected by the rat-flea.

TABLE V.

Showing the regional frequency of buboes in various groups of rats.

	Naturally infected.	Experimentally infected by fleas.		Experimentally infected by feeding.
		In cages.	In godowns.	
Rats examined	5,188	117	167	119
Rats without buboes ..	16 per cent.	18 per cent.	3·6 per cent.	8 per cent.
Rats with cervical bubo..	72 ,,	61 ,,	88 ,,	36 ,,
Rats with axillary bubo..	23 ,,	22 ,,	2·4 ,,	1 ,,
Rats with inguinal bubo..	18 ,,	35 ,,	2·4 ,,	1 ,,
Rats with mesenteric bubo	0 ,,	0 ,,	0 ,,	72·5 ,,

TABLE VI.

Showing the regional frequency of buboes in various groups of guinea-pigs.

	Infected in plague houses.	Infected in Godowns.	Infected in Sion village.	Experimentally infected in the laboratory by fleas.
Guinea-pigs examined ..	87	253	36	108
Guinea-pigs without buboes	1 per cent.	1 per cent.	2·3 per cent.	1 per cent.
Guinea-pigs with cervical bubo	92 ,,	92 ,,	92·5 ,,	91 ,,
Guinea-pigs with axillary bubo	1 ,,	3 ,,	0 ,,	6 ,,
Guinea-pigs with inguinal bubo	14 ,,	13 ,,	7·5 ,,	22 ,,
Guinea-pigs with mesenteric bubo	0 ,,	0 ,,	0 ,,	0 ,,

THE MECHANISM OF THE TRANSMISSION OF PLAGUE BY THE RAT-FLEA.

Factors that determine the Chance of Infection of the Rat-Flea.

When the Commission had discovered the conditions that made it easy to transmit plague from one animal to another by the agency of the rat-flea, they were still faced with problems concerning the fate of the plague bacillus in the body of the rat-flea and the mechanism of transference of the infection. They showed that the chances of a rat-flea becoming infected during an epizootic of plague are considerable. The average capacity of the rat-flea's stomach is about 0·5 c.mm., and therefore a flea that imbibed the blood of a rat with 10,000 or more plague bacilli per c.cm. would take at least 5 bacilli into its stomach. They estimated that 66 per cent. of plague rats in Bombay, either before or immediately after death, contained more than 10,000 bacilli per c.cm. of the blood; there are thus ample chances for the ingestion of bacilli by the rat-flea.

The Fate of the Plague Bacillus in the Flea, and the Process by which the Flea conveys the Infection to its Mammalian Host.

Infected fleas fed daily on healthy animals remained infective for a good many days, and often showed numerous plague bacilli in the stomach; it was evident that the bacilli were able to multiply in the body of the flea in spite of the washing-out process with clean blood from its host. Plague bacilli were often present in the rectum and fæces of fleas taken from plague rats, and the fæces were infective to guinea-pigs both by cutaneous and subcutaneous inoculation; the bacilli were never found in the body cavity of the flea or its salivary glands.

The Commission investigated various possible modes of infection by the flea. They found no evidence in favour of infection by contaminated mouth parts or regurgitation from the stomach; and they were unable to state whether infection by the fæces was the usual process or not. Swellengrebel (1913), in Java, never observed *X. cheopis* and *P. ahalæ* defæcating when sucking blood; working with Strickland he found this to be true also for *C. fasciatus*. He pointed out that the fæces of *X. cheopis* and *P. ahalæ* are unusually sticky, so that the chance of the skin being soiled in this way appeared to be slight. He fed infective fleas through gauze placed on the intact skin of guinea-pigs, and took care to obviate infection by fæces; his experiments, 3 in number, were all positive. He mentions that Otten in the same way, using rats as the experimental animal, obtained 4 out of 5 positive results with *P. ahalæ*. Swellengrebel concluded that the bacilli were introduced by the proboscis of the rat-flea, but that the exact mode of their introduction was unknown. Kato (1914) came to a similar conclusion.

The Observations of Bacot and Martin on the Process of Infection in the Rat-Flea.

Bacot and Martin (1914) gave a convincing demonstration of the nature of the infective process in the flea and the mode of transmission of the infection by the flea. They obtained a supply of infected rat-fleas, *C. fasciatus* and *X. cheopis*, from infected mice kept in a flea-proof cage. Mice, though less susceptible than white rats, often show an intense septicæmia before death; the bacilli in many instances are equal in number to the blood corpuscles. The fleas were segregated and the fæces of each were examined daily for plague bacilli. Twenty fleas, certified as plague-infected, were fed on a white rat on two successive days: 9 rats in 13 experiments died of plague. It was noticed that while some of the fleas sucked energetically and persistently no blood entered the stomach, and that the œsophagus became unusually distinct. These abnormal fleas were specially marked, and when dissected the proventriculus, a valvular structure situated between the œsophagus and stomach, was found to be blocked with a solid culture of plague bacilli, and the œsophagus to be more or less distended with fresh clotted blood. The obstruction, as seen under the microscope, had a dark brown colour which was due to the alkaline hæmatin resulting from the growth of the bacilli. When only 2 or 3 fleas were used for each experiment obstructed *X. cheopis* transmitted plague in 4 out of 4 instances; and obstructed *C. fasciatus* in 1 out of 6 instances. These experiments showed that the chance of infection with the abnormal fleas is high: it may be added that both male and female fleas can transmit plague.

In order to understand the sequence of events which brings about the obstruction, it may be recalled that in the rat-flea, the mouth is situated at the attachment of the appendages which form the piercing organ and which also functions as the sucking tube. The pharynx with its muscles, which are attached to the exo-skeleton of the head, operate as a pump, for by the co-ordinated action of the muscles and by the elastic recoil of the walls of the pharynx the blood is sucked from the puncture-wound and driven back along the œsophagus and through the proventriculus into the stomach. The proventriculus consists of seven rows of curved chitinized spine-like epithelial cells arranged radially, which when at rest are open for the free passage of blood, but which close when the muscular bands encircling them contract. This valve-like action prevents the blood from being propelled into the œsophagus by the vigorous peristaltic contractions of the stomach during digestion. The lodgment and growth of the bacilli in the intercellular recesses of the proventriculus forms the initial stage of the blockage, which during a subsequent feeding-act may lead to regurgitation of infected blood into the puncture-wound. The stomach of a recently infected flea shows clusters of specks stained brown by adsorbed hæmatin, which consist of plague bacilli. Later, the stomach and proventriculus show definite brown jelly-like masses, which are really solid bacterial cultures. Owing to its gelatinous consistency the culture-mass not infrequently leads to incompetence and even to complete blocking of the valve. The plug of culture may extend into the œsophagus. When this happens fresh blood cannot enter the stomach, but as the flea is thirsty it persists in sucking and thus the œsophagus may distend with fresh red blood, which may be driven back into the wound by the elastic recoil of the œsophageal and the pharyngeal wall. Hence plague bacilli may be inoculated at the same time. At first the block may be quite transient or it may be a partial one, for there may be a passage through the centre of the plug in the proventriculus—a specially dangerous condition because then contaminated blood may extend all the way from the posterior portion of the stomach to the anterior chamber of the pharyngeal pump (Bacot, 1915[2]). Blocked fleas, if they are kept cool and moist and regularly fed, will live for many days, since autolysis of the culture-mass re-establishes the passage; the obstruction may, however, recur. Bacot's observations showed that blocked fleas can live for 50 days at from 10 to 15° C., and for 23 days at 27° C.

Observations on the occurrence and frequency of blocked fleas under natural conditions during both the epidemic and the non-epidemic season do not seem to have been made. The importance of this morbid process in the flea as an effective medium for conveying the infection is indicated in some experiments carried out by the Plague Research Commission in Bombay (1907). They performed 67 experiments with white rats in flea-proof cages: one rat-flea, taken from a septicæmic plague rat, was put into each cage. Although about 50 per cent. of these fleas were estimated to contain plague bacilli in their stomachs there was only one positive result.

In connection with their work on infection in the flea, Bacot and Martin made observations on the virulence of the bacilli in the stomach of infected rat-fleas as compared with that of bacilli in the spleen of a plague rat. White rats were carefully shaved over part of the abdomen. Three days later, 20 normal rat-fleas were fed through gauze for about one minute. Immediately afterwards the cut surface of a plague spleen was gently applied to the shaved area in a group of rats ' A '; the area in a group ' B ' was treated with a strong suspension of bacilli from fleas' stomachs which were full of bacilli; and the area in group ' C ' was treated as in group ' A ' but without the flea punctures. Nine out of 10 of the group ' A ' rats died of plague; 5 out of 23 of the ' B ' group rats died of plague; and none of the ' C ' group were infected. The belief of these workers that the difference of the results in groups 'A' and 'B' was due to a difference in virulence was confirmed by phagocytosis experiments. The average uptake of the polymorphonuclear leucocytes of their own blood was 3 to 5 flea-stomach bacilli, whereas no spleen bacilli were taken into the leucocytes.

Observations on the Duration of the Infectivity of Rat-Fleas.

Most of the experiments bearing upon this question have been done under conditions that do not resemble those met with in nature. For example, the opportunities for feeding presented to the flea affect the growth of the bacillus in the alimentary canal. But the fleas in most of the experiments that have been recorded have been regularly fed; in nature, the rat-flea, when its host dies of plague, is likely to be deprived of regular meals. Again, the temperature and moisture of the air in which the flea finds itself, especially when starved, influence to a large extent its chances of survival.

The Plague Research Commission (1908) studied the factors that operate upon the infective process in the flea. They found that a proportion of fleas are able to rid themselves of bacilli, and that the clearing mechanism is probably a phagocytic one. Experiments showed that a rise of temperature of about 8° C. between the limits 24 and 32° C. doubles the rate at which the bacilli disappear; Ledingham had shown that for a rise of 10° C. the rate of phagocytosis is increased about 2·2 times. Infected fleas fed on immunized animals were just half as capable of infecting guinea-pigs as were fleas that had fed on susceptible animals: a further proof that phagocytosis is an important factor.

The Commission (1907) fed infected fleas taken from plague rats during the plague season on a fresh healthy animal each day thereafter. Numerous plague bacilli were seen in the stomach contents of 76 out of 302 fleas (25 per cent.) that were dissected from the first to the twelfth day; and of one flea when examined as late as the twentieth day. In the first four days after the fleas had fed on septicæmic blood, 43 per cent. had numerous plague bacilli in the stomach; and from the fifth to the twelfth day 13 per cent. were still infected. In a second series of observations made between the 7th of May and the 9th of June, 1906, when epidemic plague had almost died down in Bombay, the percentage of infected fleas was very much smaller, and the seventh day was the limit of the infective period. The percentage of fleas found to be infected was six times greater in the epidemic season than in the non-epidemic season. The Commission (1907) showed also that during the epidemic season rat-fleas, fed on a septicæmic rat, and fed daily thereafter on a healthy animal, remained infective for at least 10 days: this series of experiments was made in separate cages with a small number of fleas. A second series carried out in a similar way but in a large flea-breeding cage with abundant rat-fleas gave an infective period of 15 days. In a third series of experiments, similar to the second, but performed during the non-epidemic season, the fleas were infective for only 7 days, and fewer animals contracted the disease.

Swellengrebel (1913) carried out similar experiments with *X. cheopis* in Java; at Malang the fleas remained infective for 28 days at a temperature of 23° C., and a relative humidity of 76 per cent., and at Surabaya for 19 days. Swellengrebel, therefore, thought that the freedom of Surabaya from plague had no relation to flea infectivity, but, as already stated, the conditions of his experiments were perhaps somewhat artificial: it would be better in similar experiments to ascertain the effect of the local atmospheric conditions on starved blocked fleas. In one experiment, he transmitted plague by fleas which had fasted for 6 days. The limit of infectivity in Otten's experiments in Java (1917) was 43 days; in Hoesen's experiments (1923), made also in Java, it was 33 days.

The duration of infectivity of C. fasciatus. Raybaud (1911) states that Gauthier and he had found virulent plague bacilli in the intestine of infected *C. fasciatus* after storage in the ice-chest for 45 days. Bacot (1915[3]) fed from 100 to 300 hungry *C. fasciatus* on plague mice when these were in the comatose condition which immediately precedes death. Stomach smears of samples of each batch showed that from 65 to 85 per cent. of the fleas carried *B. pestis.* Cages were stocked with these infected fleas, covered with a waxed cloth to check drying, and stored at temperatures varying from 1·7 to 15·5° C., the average being 7·5° C. At intervals, the cloth was removed and two healthy mice were added to each cage: of 8 cages tested, 3 remained infective, namely for 29, 34 and 47 days respectively.

The Question whether the Plague Bacillus passes through the Larval Stage of the Flea to the Adult Insect.

Bacot (1914[1]) showed that flea larvæ of *X. cheopis* and *C. fasciatus* thrive on a diet of their parents' fæces, which often contain *B. pestis.* He thereupon (1914[2]) tested the possibility of the bacilli passing through the developmental stages by feeding newly hatched larvæ of *C. fasciatus* and *P. irritans* on fresh blood heavily contaminated with virulent *B. pestis.* The plague bacilli were found not to pass from the larval to the pupal or imaginal stages. Moreover, larvæ of *C. fasciatus* taken from the bodies of mice dead of bubonic plague, whose hair was thickly speckled with the droppings of infected fleas, were dissected and smears made of the stomach contents: no trace was seen of the multiplication which is so characteristic of the infected adult insect.

THE RELATION BETWEEN THE EPIZOOTIC AND THE EPIDEMIC.

The Observations of the Plague Research Commission in India (1907, 1912).

The chart published in the Report of the Commission's work summarizes in graphic form their observations on rodent and human plague during the year from October, 1905, to September, 1906, inclusive, in Bombay City. The figures from which the curves were drawn are considerable: thus, during that period more than 4,000 *M. rattus* and 13,000 *M. norvegicus* were shown to be plague-infected; and there were 11,000 plague deaths. The chart conveys at a glance the relations of the epizootics to the epidemic. First in order of time comes the curve which represents the *M. norvegicus* epizootic; this is followed at an interval of about 10 days by the epizootic curve for *M. rattus;* and this again at an interval of from 10 to 14 days by the epidemic curve. The precedence of the *M. norvegicus* epizootic is accounted for by the circumstance that the average number of fleas on *M. norvegicus* was more than double the average number on *M. rattus;* and in agreement with this finding the incidence of plague upon *M. norvegicus* was twice as great as upon *M. rattus,* although laboratory experiments showed that there was no difference in their susceptibility to plague by flea transmission. The interval of about 10 days which separates the *rattus* curve from the *norvegicus* curve corresponds fairly well with an average interval, in 30 successful flea-transmission experiments with *M. rattus*, of 7·2 days, which elapsed between the date of exposure to the bites of infected fleas and the deaths of the rats. The Commission divide the 10 to 14 days' interval between the *rattus*-epizootic and the epidemic curves into the following periods :

(1) The starvation period after which the rat-flea bites man most readily ..	3 days.
(2) Incubation period of human plague ..	3 ,,
(3) Duration of illness of fatal human plague	5½ ,,
	11½ ,,

This estimate is in fair agreement with the chart.

The statistics of plague in man and in rats from 1907 to 1911 in Bombay confirm the original conclusions (1912). Greenwood's statistical analysis of the Commission's figures for the period 1905–6 shows a high correlation between the incidence of plague in man and in *M. rattus*. *M. rattus* in Bombay, as elsewhere, lives in far closer association with man than *M. norvegicus*.

The Observations of Other Workers.

There are no records on a similar scale for other countries, although it is stated that figures extracted from the Hong-Kong reports for 1895–1918 reveal agreement with the Bombay results, the curve for rodent plague preceding by a short interval that of the epidemic (Petrie and Todd, 1923). None the less, plague workers are generally agreed that there is a causal connection between plague in rats—and especially *M. rattus*—and human plague.

Swellengrebel and Hoesen (1915[1]) have discussed the question as it relates to Java. They admit that statements to the effect that human plague may occur without rat plague are not convincing, and point out that when plague broke out in Java, plague rats were not at first found in the infected villages, because it was not then known that the rats died in their nests in the hollow bamboos of which the native houses are constructed.

In the opinion of the present writer, statements that human plague may appear in the absence of rodent plague are valueless unless it is shown that a thorough search for rat-fleas has been made by means of guinea-pig traps. The village of Atmour Mistigid on the Komombo Estate near Aswân in Upper Egypt is the best instance known to him of a severely plague-infected locality with no discoverable evidence whatever of rat plague, nor even of rat infestation, and yet with a very heavy infestation of the houses with *X. cheopis* (Petrie and Todd, 1923). There are a number of references in the literature which ascribe the spread of small outbreaks, apparently independent of rodent plague, to the human flea or to bugs. The localized outbreaks in South Africa, now known to have their origin in the veld rodents, gave occasion for an error of this kind.

Swellengrebel and Hoesen (1915[1]) state that in Java rat plague may exist in the villages in the absence of human plague: their observations seem to have been made during the interepidemic months. The method adopted for the discovery of plague in rats was the inoculation of their spleens, which were ground up together, and of their fleas, which were treated in the same way. Thus in the period from June to December, 1913, 47 villages in the province of Kediri were searched for infected rats; evidence of their presence was obtained in 18 instances by means of the inoculation of material from 15,279 triturated fleas; the fleas had been taken from 3,230 rats. By the inoculation of spleen emulsions prepared from 7,265 apparently healthy rats that were caught alive, evidence for the existence of foci of rat plague was found 54 times. In 11 of the 47 villages there was plague in rats, but not in man. It is perhaps fair comment to say that the rigour of these methods for detecting rat-plague necessarily went beyond the methods available for diagnosing human plague; and that sporadic cases of human plague are easily overlooked in primitive communities. Nevertheless, the observations are of interest because, as the authors themselves realized, they render needless the supposition that a form of chronic plague in rats or of *pestis minor* in man carries the infection over the non-epidemic period.

Swellengrebel and Hoesen believe that the occurrence of human plague following rat plague is conditional upon the number of rat-fleas which become free at a particular time, and upon the number of living rats which, at the same time, are available for the fleas to attack. If the rat-fleas are numerous and their hosts few, the conditions are favourable for the occurrence of human plague.

Swellengrebel (1913) has suggested that the incidence of trypanosomiasis in rats—a flea-borne infection—is a useful warning of the presence of plague, and that a low value for the ratio between the percentages of rats carrying trypanosomes and fleas respectively, may signalize an outbreak of plague. He thinks that plague infection inhibits the development of trypanosomes in the rectum of the flea so that fleas that are infected with plague will not be able to transmit trypanosomiasis. This relationship has not so far been confirmed.

When instances are given of the occurrence of rat plague without human plague, or conversely, the species of rat or other rodent should be mentioned, and also the species of the infesting fleas.

The Species of Flea that are able to Transmit Plague.

X. cheopis.

In recent years the number of species that have proved to be plague-carriers has increased, but none has displaced *X. cheopis* as the chief transmitter of the infection. In India, in Java and in Egypt this species is the predominant one. Hirst (1926, 1927) has not been able to trace a single instance of bubonic plague becoming endemic among the rats in any locality in the absence of *X. cheopis*. This author states that in Colombo, storage of grain, the prevalence of *X. cheopis* and the incidence of plague are interrelated. He regards *X. cheopis* in Colombo as an imported flea, and he has been able to trace sporadic cases of rat and human plague to recently imported grain or gunny bags; rats caught in such goods yielded from 50 to 100 per cent. *X. cheopis*. Fleas on ship-rats seem to be mostly *X. cheopis*, and a higher percentage of this flea is obtained from rats in warehouses in harbour areas than farther inland.

The plague history of Australia shows that New South Wales and Queensland were the worst infected States from 1900–9, and that the rats there had a high infestation with *X. cheopis*; in Victoria, which was only slightly affected, the rats had a minimal *cheopis* infestation. Tasmania has remained quite free from plague, and it is significant that *X. cheopis* has not been found there; in Hobart all the fleas taken from rats have proved to be *C. fasciatus*; *Ctenopsylla musculi* appears to be the common rat-flea of Tasmania (Cumpston and McCallum, 1926).

In New Orleans, where plague has been prevalent, *X. cheopis* is the principal rat-flea during every month of the year, and *C. fasciatus* is practically absent, but in New York City and in Boston, where plague has never existed, *C. fasciatus* is the chief rat-flea, *X. cheopis* having been found in considerably smaller numbers in most months of the year (Fox and Sullivan, 1925).

X. astia.

In 1912, two years before plague broke out in Colombo, Hirst collected about 400 rat-fleas during the plague season in the centre of the area where *X. cheopis* is now the most prevalent flea, and where also plague chiefly appears. All these fleas were identified as *X. astia* by Rothschild. Rat plague first appeared in February, 1914, in this area, and in the following month 16 of 199 rat-fleas collected here were found to be *X. cheopis*; this species has since been the prevalent one in the plague area of Colombo. Hirst, in 1913, discovered that the rat-fleas in Madras, a city noteworthy for its freedom from plague, were purely *X. astia*, and in consequence of his observations he originated the theory that local prevalence of *X. astia* is co-related with local immunity to plague. The results of experiments recently published by Goyle (1928) indicate that *X. astia* is inferior to *X. cheopis* as a transmitting agent of plague to Indian rats, but on the other hand, Mital's observations (1928) on the distribution of *X. astia* and *X. cheopis* in the United Provinces leave the question of their relative importance still uncertain. Further work on the subject is needed before a final conclusion can be drawn.

X. brasiliensis.

In 1914, Rothschild showed that Indian rats were infested by three species of *Xenopsylla*: *X. cheopis*, *X. astia* and *X. brasiliensis*. The last-named species is well established on rodents throughout Peninsular India, but no detailed information exists regarding its bionomics, its relation to human plague, and its ability to convey plague experimentally (Hirst, 1926, 1927).

Ceratophyllus fasciatus.

In the experiments of Bacot and Martin on the mechanism of transference of plague, infection appeared to be more easily conveyed by *X. cheopis* than by *C. fasciatus*, and obstruction of the proventriculus by plague bacilli certainly occurred more readily in *X. cheopis*. Two transmission experiments on guinea-pigs were successful with a species of *Ceratophyllus* found by the Plague Research Commission (1907) on the rats in the villages of Kasel and Dhand in the Punjab and supposed to be *C. fasciatus* ; this species has since been identified as *C. punjabensis*.

Other species of fleas on rats and other rodents.

In Java, in South Africa, in Japan and elsewhere, plague-carrying rodents are infested with various species of fleas, some of which have been shown to transmit plague experimentally. An account of them need not be given here, because the knowledge we possess of their plague-transmitting powers does not differ materially from that which has been gained as the result of more extensive observations on *X. cheopis*.

THE SEASONAL PREVALENCE OF BUBONIC PLAGUE.

The Influence of Temperature upon the Infectivity of the Rat-Flea.

Proof has already been adduced that the rat-flea was the infecting agent in the experimental epidemics described by the Plague Research Commission. The spread of the infection among the rats and guinea-pigs in these epidemics followed a course which corresponded with the prevalence of rat plague and human plague in Bombay City; the epidemics during the plague season ran a rapid course with high mortality, whereas those in the non-epidemic season spread much more slowly, and most of the animals escaped the infection. In the Commission's view the high temperature was the limiting factor in the spread of the epidemics. The severe and rapid epidemics occurred when fleas were abundant and the temperature ranged between 21 and 24° C. (70 to 75° F.).

A much higher percentage of successful flea-transmission experiments with Bombay wild rats, and also with guinea-pigs, was obtained during the plague season than during the off-season. Experiments of this kind were made in July–August, 1906, that is, during the off-season ; (1) at room temperature, with an average of 27 to 28° C. (80 to 83° F.), and (2) in a specially constructed cool room kept at 21° C. (70° F.). Bombay rats, ship-rats and guinea-pigs were used, and the proportion of successful transmissions at 21° C. was nearly twice that at 27·5° C.

Similar experiments were made in January–February, 1907, that is, at the beginning of the plague season, (1) at room temperature, namely, 24° C. (75° F.), and (2) in a specially constructed hot chamber kept at from 29·5 to 32° C. (85 to 90° F.); guinea-pigs were used and the ratio of successes was 84/32, or nearly 3 to 1 in favour of the lower temperature.

Transmission experiments were successfully carried out with guinea-pigs and fleas at low temperatures, namely, 4·5° C., 10° C. and 15·5° C. (40° F., 50° F., 60° F.), but it should be observed that only fleas taken from rats with a high degree of septicæmia were used in the experiments. In the hot season fewer fleas were found to be infected, the bacilli disappeared from the stomach much more quickly, and the fleas lost their power of infecting animals much sooner than in the cold season.

In the experiments mentioned above, which were carried out in January–February, 1907, the infectivity of the fæces of the rat-fleas was tested by inoculating guinea-pigs subcutaneously with an emulsion in saline. The fæces of fleas kept at room temperature, namely, 24° C. (75° F.) were infective till the twenty-first day, whereas the fæces of fleas kept at 32° C. (90° F.) were infective for only 3 days. The higher temperature obviously assists the cleansing of the alimentary canal from plague bacilli.

The Commission found that both in Bombay City and in the Amritsar district of the Punjab there was a distinct seasonal variation in the number of rat-fleas, which corresponded in a general way with the plague mortality. Bacot and Martin (1924) tested the effect of temperature and moisture on the survival of the rat-flea away from its host, and concluded that the survival rate was approximately in inverse ratio to the saturation deficiency of the air, when the temperature and air movement were kept constant, in other words, it was proportional to the rate at which the flea lost water. With a constant saturation deficiency its length of life was reduced to between half and two-thirds by a rise of 10° C. They point out that the effects of temperature and drying reinforce each other, and that they may throw light upon some of the climatic factors in the Indian epidemics. These authors think that hot dry weather must shorten the life of blocked fleas. Brooks's (1917) analysis, on behalf of the Advisory Committee on Plague in India, of data relating to various localities in India and elsewhere, led him to conclude that a critical saturation deficiency exists for each range of temperature, and that it has an effect, together with the temperature factor, in shaping the course of an epidemic, and particularly in bringing it to an end. Poona is an instance of a locality where the temperature is never really unfavourable to the spread of plague, and where the relative humidity of the air, by its action on the multiplication of fleas, is the controlling factor.

A study of the climatic factors implicated in the seasonal prevalence of bubonic plague, which was undertaken with the purpose of throwing light upon the gradually increasing delay in the onset of epidemic plague from south to north of Egypt, indicated that there is an optimum range of temperature from 20 to 25° C., and a saturation deficit of water vapour of from 1 to 10 millibars for plague prevalence; and that the developmental stages of the rat-flea proceed best within these limits (Petrie and Todd, 1923).

Swellengrebel (1915) refrains from criticizing the conclusion of the Plague Research Commission that the flea average is an important factor in the seasonal rise of the epizootic, and states that in Java this relation cannot always be observed; here the increase is probably not the cause but the consequence of the epizootic, since a heavy mortality among rats is likely to bring about a concentration of fleas upon the survivors. That, nevertheless, there is a seasonal increase in the number of fleas on rats where plague is absent is clear from observations made at Asyût in Egypt; the curve corresponded well with that of the incidence of human plague in the surrounding district (Petrie and Todd, 1923).

To sum up, the seasonal prevalence of human bubonic plague is dependent upon the prevalence of rat-plague, and this in turn is governed by the climatic factors—temperature and relative humidity—which influence the developmental stages of the rat-flea and the multiplication of the plague bacillus within the rat-flea.

THE TRANSPORTATION OF THE INFECTION IN THE RAT-FLEA.

The Plague Research Commission (1907) tested this question in Bombay City by collecting bundles of clothing and bedding from houses in which plague cases had occurred. The bundles were opened out, kept in a flea-proof godown for several days, and then replaced by fresh lots. Twenty-six free-running guinea-pigs were kept in the godown for an average period of about 4 days each, with the result that 3 fleas—2 rat and 1 human—were caught on them and that one of the guinea-pigs died of plague; the bubo was in the neck. This guinea-pig apparently died of flea-borne plague, because a control experiment with infected bedding obtained from a plague hospital and free from fleas caused no infection. The experiments were carried out at an unfavourable time, namely, at the end of an epidemic. In the writer's opinion they should be repeated on a larger scale, because there is a sharp difference of opinion among plague workers on the mode of dispersal of infection over considerable distances.

The Committee's epidemiological evidence indicated that infection could be imported into hitherto uninfected villages by rat-fleas conveyed in the clothing or personal effects of visitors who, nevertheless, might escape the infection. They thought that the house-rats became infected from such imported rat-fleas. Swellengrebel (1913) examined the parasites collected from the clothes and baggage of travellers who were departing from the Province of Malang, and found only 2 *X. cheopis* and 1 *P. ahalæ* among the insects taken from 56,790 men, who did not all come from infected villages. The clothes of 1,829 persons, among whom were 393 suffering from plague, yielded 7 *X. cheopis*. He believes, therefore, that the transport of fleas is a minor factor in the spread of plague in Java. Flu [cited by Hirst (1926, 1927)] thinks that rat-fleas, through the medium of rice, play a more important part in the spread of plague in Java than infected rats. He caught numerous *X. cheopis* on guinea-pigs placed among bags of rice. Otten (1923) thinks that the native traveller in Java is not an agent in transporting rat-fleas, but suspects the marketing villager who carries about foodstuffs or vegetable produce for barter or sale. Some of these foodstuffs are stored for months and may serve as food and as a nesting-place for the house-rat, and in this way infected rat-fleas can be carried from an infected centre to villages at a distance. Otten regards human traffic, in the widest sense, as the only medium for the dissemination of plague to near and far distances.

The authors of the Report on Plague in Egypt (Petrie and Todd, 1923) incline to the view of the Plague Research Commission in India. They examined the clothes of 4 plague patients in a badly infected village on the Komombo Estate. Only 2 *P. irritans* were found in the 4 lots of clothes, and the numbers of *X. cheopis* were 4, 4, 4 and 1; one of the sets of clothes belonged to a man who, when detected, had walked 5 miles in order to escape quarantine. Further, 185 *X. cheopis* were taken from the clothes of 3 rat-catchers after they had dug out nests in houses in the village. Creel (1914 and 1916) has criticized the point of view of the Plague Research Commission on the flea-borne extension of plague, but has apparently misunderstood it. He attributes to the Commission the idea that a human distributor of rat-fleas may escape infection from them, but that they may directly infect a person in a house at which the visitor makes a stay; as already stated, the Commission held that the imported fleas are likely to attack, in the first place, the rodent inhabitants. Creel believes that the diffusion of plague within towns results chiefly, if not entirely, from the migration of infected rodents; and that the transference of fleas in merchandise is improbable. It seems clear that his experience in New Orleans, Cuba and Porto Rico has been different from that which has been gained in countries like India, Java and Egypt.

Hirst (1926, 1927) does not doubt that infective *X. cheopis* may be carried for considerable distances on human beings or in their personal belongings. He points out that in Colombo there is a constant danger of re-importing the infection from Rangoon, which is becoming the principal focus of plague infection among the ports of the East. In Colombo the association between imported grain and plague is particularly clear, and in this city grain is the chief vehicle of infection. The greatest danger probably arises from the importation of grain and of gunny bags that are heavily infested with the eggs and larvæ of *X. cheopis* owing to storage in rat-infested warehouses in the place of export. If the distance between the ports of despatch and receipt is short, an interchange of flea species may take place in this way, but when the distance is long, ship-rats are likely to be the carriers and distributors.

THE MODE OF INFECTION OF MAN IN EPIDEMICS OF PLAGUE.

Evidence which Disproves the View that the Infection may be Spread by Contamination with the Excreta of the Rat or of Man.

During the early years of their work the Plague Research Commission in Bombay tested the view, then widely current, that, as plague in the rat and man is often a septicæmic disease, the excreta are likely to contain the bacilli and thus help in distributing the infection. Their work yielded no evidence whatever in support of this view, so that only a brief summary of their conclusions need be given.

1. Floors, constructed of materials used in the native houses, when grossly contaminated with plague cultures, remained infective for less than 24 hours to guinea-pigs which were allowed to run freely about on them.
2. The urine of 32 per cent. in one series and of 19 per cent. in another series of patients suffering from septicæmic plague was infective to guinea-pigs, when inoculated subcutaneously, *but in no instance did guinea-pigs inoculated cutaneously with the urine contract the disease.* The urine contained far fewer bacilli per c.cm. than the blood. The infectivity of the fæces in human septicæmic plague is negligible.
3. Guinea-pigs remained healthy when subjected in a confined space to intimate and prolonged contact with soiled linen and bedding which had been removed from hospital patients just before their death.
4. The urine of human plague patients was given in food to Bombay rats ; 194 rats were each given 2 or 3 c.cm. of the urine mixed with food ; not one of them contracted plague.
5. The blood of Bombay rats may contain from 10 to 100 million plague bacilli per c.cm., but the urine does not contain even 10 bacilli per c.cm. ; the fæces are seldom infective when inoculated cutaneously into fresh rats.
6. Wild Bombay rats contract the intestinal form of plague when fed on infected material ; a mesenteric bubo was present in about 75 per cent. of the rats and a cervical bubo in about 35 per cent. In many thousands of *naturally infected* rats examined by the Commission not one with a mesenteric bubo was discovered.

The Possibility of the Transmission of Plague by Insects other than the Rat-Flea.

P. irritans. In recent years there has been a tendency to attribute to the human flea an important part in the spread of some epidemics, but the evidence adduced in support of the idea is not convincing. It is obviously necessary to seek first for evidence of rat-flea infestation in the houses of patients by trapping for rats, and, if the search fails, by using guinea-pigs as flea-traps.

The Plague Research Commission (1906) noted that of 85 human fleas caught on 'Tanglefoot' in plague houses, only 1 contained plague bacilli, whereas of 77 rat-fleas thus caught, 23 were infected. Human fleas do not live well on wild rats; after 24 hours, only 1·2 per cent. of human fleas put into cages with wild rats were recovered alive.

Attempts were made to transmit plague experimentally by means of the human flea. About 20 fleas that had previously been fed on selected septicæmic rats, and were, therefore, presumably infected, were put with a guinea-pig in a flea-proof cage. Thirty-eight experiments of this kind were performed in April and May, 1906, and only 3 guinea-pigs became infected. The process of infection in the human flea does not seem to have been investigated in the light of the observations of Bacot and Martin, but in the Commission's experiments fleas with a large number of plague bacilli in their stomach were found up to the fourth day.

Bubonic plague rarely spreads by direct contact, a statement supported by hospital experience and by the circumstance that multiple cases in houses are infrequent, and that when they do occur the attacks are often almost simultaneous, as if they were due to a common infecting agent. The Commission, taking these considerations into account, and having regard to the slight septicæmia in human plague as compared with that in rats, thought that transmission from man to man by the human flea was a rare occurrence. Observations on the prevalence of human fleas were made from February to November inclusive, in Upper Egypt; they showed a high degree of infestation during the plague season, and yet there was nothing in the epidemiological features of plague at that time in Egypt to indicate that the human flea took any share in its spread (Petrie and Todd, 1923).

Bugs. The work of Bacot (1915[1]) on the experimental transmission of plague infection by the bug, showed that a meal of septicæmic blood from a mouse is fatal to a proportion of *Cimex lectularius*, and that the survivors, even after 48 days' starvation, may infect a mouse. The mechanical blockage, which is the characteristic feature of infection in the flea, is precluded in the bug by the absence of a valve between the pharyngeal pump and the œsophageal crop. Cornwall and Menon (1917) were able to infect bugs, but a large proportion of them died in a few days, and they failed in their attempts to transmit plague to guinea-pigs by means of these insects. They think that plague is unlikely to be conveyed to man under natural conditions by bugs in the act of biting.

Lice. Lice (*P. hominis* and *P. capitis*) from plague patients have been found by a few observers to contain plague bacilli, but epidemiological evidence discounts the view that lice commonly convey the infection.

Mosquitoes. Under experimental conditions mosquitoes can be infected, but they are unable to transmit plague to guinea-pigs (Flu, 1917).

The Rodent-Flea as a Transmitting Agent of Plague to Man.

X. cheopis readily bites man, especially if it is left without food for 24 hours or longer; it has been kept alive for 25 days when fed on man alone. Rat-fleas have been taken from the limbs and clothing of men who have entered plague houses. Although direct evidence naturally cannot be obtained, the experiments that have been surveyed in this chapter are consistent with the belief that man is infected by the rodent-flea; they form a mosaic of evidence which is virtually conclusive. There is no feature of the spread of bubonic plague that is not explicable on the theory of transmission by the rodent-flea. Sanitarians in plague-infected countries everywhere have accepted the theory, and have founded their preventive measures upon it.

Natural Plague in Rodents and in Other Animals.

PLAGUE IN RATS.

The Diagnosis of Acute Plague in Rats.

The diagnostic significance of the post-mortem signs.

Macroscopic signs. The first adequate account of the lesions in plague rats—*M. rattus* and *M. norvegicus*—was given by the Plague Research Commission in India (1907). They regarded a typical primary bubo as the most important sign; 75 per cent. of the buboes were in the neck. Plague workers everywhere have failed to find a mesenteric bubo in a naturally infected rat. Records from other countries of the distribution of buboes in rats are not comparable with those of the Commission, because each worker has followed his own idea of what constitutes a bubo. The detailed description of this lesion given by the Commission might well serve as a basis for systematic record. The granular appearance of the liver due to the presence of small areas of necrosis scattered over the surface is a characteristic sign; it was seen in 55 per cent. of the rats in Bombay. General subcutaneous congestion, hæmorrhages either in the subcutis or in the organs, and an abundant clear pleural effusion are other noteworthy signs. The spleen is firm and has a moulded appearance; granules were present in it in 4·5 per cent. of the rats. The lungs showed necrotic granules in only 2·5 per cent., and a typical primary plague pneumonia was seen in 0·6 per cent. of the rats.

The features of greatest service for diagnosis as exhibited by 35 plague-infected *M. norvegicus* examined by Macalister and Brooks (1914) were subcutaneous congestion, clear pleural effusion, and granular necrotic changes in the liver; all three were present in most of the rats, and no rat failed to display one or more of these signs. The collective significance of the lesions should especially be considered.

The following differences exist between the lesions of naturally infected rats and of plague-infected guinea-pigs: (1) Subcutaneous hæmorrhages are common in rats; they are rarely seen in guinea-pigs. (2) A general subcutaneous œdema is rare in rats; but it is a very characteristic feature of experimentally infected guinea-pigs. (3) Subserous intestinal hæmorrhages are rarely present in rats; but they are a common and striking feature in guinea-pigs. (4) The liver is the important organ for diagnosis in rats; the spleen in guinea-pigs.

Microscopical examination. Carbol-thionin blue is a useful stain for smears. Involution forms were noted in 57 per cent. of the primary buboes in the Bombay rats, but in only 12 per cent. of the spleen preparations; they were never observed in the heart-blood. The bubo gave a better chance of finding plague bacilli than the spleen, and the spleen than the heart-blood. In 75 per cent. of one series of rats that were examined in Bombay, numerous plague bacilli were present in the heart-blood, the spleen or the bubo; or if there were not many in the bubo involution forms were present. Macalister and Brooks observed, as many previous workers had done, that bipolar staining is shown at times by intestinal and putrefactive bacteria; and they also found bipolar bacilli, probably of intestinal origin, in the accessory sexual glands of otherwise healthy rats. They observed also that putrefactive bacteria may simulate pleomorphic plague bacilli, especially in decomposed pleural effusions. The Plague Research Commission regarded naked-eye examination by a competent observer as being a better method for diagnosis than microscopical examination alone.

Serological diagnosis.

Kister (1906) triturated organs which contained numerous plague bacilli, and tested for specific agglutination in a hanging-drop of the organ-suspension.

Cultural diagnosis.

This is easy in proportion to the freshness of the specimens examined. Eastwood and Griffith (1914) obtained a direct culture from 29 of 35 plague-infected rats from East Anglia; many of the cultures were pure.

Identification of B. pestis *by means of special culture media.* Cultures on Hankin and Leumann's 3 per cent. salt agar medium present peculiar swollen forms, which are spherical, balloon-shaped or spindle-shaped; they may resemble yeasts or protozoa, for example, trypanosomes; these forms are best seen in cultures grown on a *dry* salt agar medium. In the experience of Dieudonné and Otto (1927), the rapid appearance, in from 24 to 48 hours, of involution forms on this medium is a character which is confined to the plague bacillus. Petrie and Macalister (1911) observed that malachite-green broth is not decolorized by *B. pestis* or only with extreme slowness, and that tubes inoculated with *B. pseudotuberculosis rodentium* lose the colour in 2 or 3 days. Eastwood and Griffith (1914) confirmed this observation; with many strains of the plague bacillus they noted a slight decolorization after about 5 weeks' incubation.

Uriarte and Villazon (1924) suggested a peptone-water medium containing glucose or lævulose, with neutral red as indicator. In from 20 to 48 hours the plague bacillus produces a beautiful yellowish colour, which becomes less marked at the end of 3 or 4 days. The liquid is transparent, and there are flocculi on the walls of the tube, but there is no formation of pellicle. Pirie (1927) discovered that in this medium *B. monocytogenes* gave reactions indistinguishable from those of *B. pestis*, but, nevertheless, he thinks the test is useful. He also gave a trial to Otten's (1926) procedure, which consists of cultivation in litmus peptone-water containing diminishing concentrations of glucose, namely, 0·5, 0·25, 0·1 and 0·05 per cent. The plague bacillus produces strong acidity in all, whereas the pseudotubercle bacillus gives feeble acidity in the 0·05 per cent. glucose tube; this later becomes alkaline. His results were in general agreement with those described by Otten, but were not so clear-cut.

Colas-Belcour (1926) grew strains of the plague bacillus and of the pseudotubercle bacillus on litmus glycerin agar at 34° C.; he states that *B. pestis* produced no change, whereas *B. pseudotuberculosis rodentium* reddened the litmus. Pirie tried this medium, and like other workers, found that some plague strains fermented glycerin. The medium has, therefore, only a relative value.

From this summary it is clear that there exists at present no culture medium that will distinguish with certainty between *B. pestis* and *B. pseudotuberculosis rodentium.*

Diagnosis by means of animal inoculations.

Any of the usual methods of inoculating a susceptible animal may be used, if the organs are fairly fresh; the cutaneous method in the guinea-pig is a useful one. The Plague Research Commission (1907) found that the course of the infection which follows this method depends mainly upon the size of the infective dose. With small doses the animals succumb late with a chronic form of plague; this holds true also of the subcutaneous method, which appears, however, to be more sensitive in responding to a small dose than the cutaneous. The local cutaneous reaction may be seen 12 hours after the inoculation; it consists of phlyctenules surrounded by a zone of redness. Thirty-six hours after inoculation there may be a well-marked skin reaction and also small inguinal buboes; in a chronic infection the glands may be palpable only after several days. Except when death occurs very rapidly, there is a loss of weight, which varies with the acuteness of the disease. The Commission found that, when fairly fresh material was used, the cutaneous method failed in only 1 per cent. of the tests.

Diagnosis of plague in putrid rats.

The recognition of plague in ship-rats, when the cadavers at the time of examination are more or less putrid, demands much time, labour and skill. Three important signs may persist: the primary bubo, the granular appearance of the liver, and the pleural effusion. Even in a very putrid rat, the primary bubo may show many plague bacilli, frequently with involution forms, and with fewer putrefactive bacteria than in the spleen and heart-blood. The Plague Research Commission in India believed the cutaneous method in the guinea-pig to be valuable ; it failed in only 10 per cent. of putrid rats. Infection by putrefactive bacteria very seldom resulted after cutaneous inoculation. They thought that the virulence of the plague bacilli was probably not diminished by association with putrefactive bacteria ; most Continental workers are, however, agreed that the virulence of *B. pestis* in the organs of putrid rats is apt to be reduced, thereby making the cutaneous test an uncertain one. Van Loghem (cited by Flu, 1924) advises liver-juice rather than spleen-juice for the cutaneous test, because, in his view, *B. pestis* in the liver resists the action of putrefactive bacteria longer than in the spleen.

Drennan and Teague (1917) sought for a medium which would make the diagnosis of rat plague easier when putrefactive changes were present. They tested the effect of a considerable number of aniline dyes, and concluded that agar with the addition of 1/700 per cent. of crystal-violet was perhaps the most effective medium of those tried. According to Markl (1913), *B. proteus* is the most disturbing associate of the plague bacillus, because, owing to its active motility, it quickly covers the whole surface of the medium, and, moreover, exerts an antagonistic action. Kister (1924) recommends the Endo medium for restraining the growth of *B. proteus*, and states that the optimal amount of fuchsin and sodium sulphite should be determined by a previous test ; the plague colonies on this medium are of normal appearance. Kister and Schumacher (1905) and other German workers showed experimentally that the chance of discovery of *B. pestis* depends upon the surrounding temperature, the number of plague bacilli in the cadaver, and the stage of putrefaction. Thus the plague bacillus was recovered after 93 days at from 5 to 12° C., and only up to 4 days at 32° C. Kister and Schumacher (1905) prefer the ' Hauttasche ' method in guinea-pigs and rats, and, as a supplementary test, give graduated amounts of organ-suspensions subcutaneously. De Raadt (cited by Flu, 1924) studied the progressive putrefactive changes in rat corpses, and was able in this way to fix approximately the interval between death and the date of examination ; with a mean daily temperature of 25·5° C., mummification was complete after 8 days.

The use of the thermo-precipitin reaction of Ascoli in the diagnosis of putrid plague-infected rats. Piras (1913) in Genoa made use of the test devised by Ascoli for the diagnosis of anthrax when only putrid material is available. The test consists of carefully adding to specific serum in a test-tube the filtrate of a boiled suspension of the organs ; if the corresponding bacterial antigen is present, a precipitation ring quickly forms at the surface of contact. Kraus (1897) had already found that plague-immune serum caused a specific precipitate when mixed with extracts of plague bacilli. Warner (1914), working in Kolle's laboratory, and a number of other workers, mostly Italian, have tested the method, and they believe that it is a useful supplement to the ordinary methods of diagnosis. Non-specific reactions may be eliminated by diluting either the extract or the serum. Careful filtration of the extract and the serum is essential; in spite of this, it is not always easy to get a clear filtrate from putrid organs. The intensity of the reaction depends upon the number of plague bacilli in the material tested, and for this reason a negative reaction does not necessarily exclude a diagnosis of plague. Warner thinks that the bubo, the spleen, and the liver, in the order named, are the most likely organs to give a positive result. Pulgher (1922) prepares the extract by adding the suspected liver and spleen to an equal volume of saline, and then boiling and filtering through paper or a layer of asbestos and of cotton-wool. A sample of serum is chosen which does not give an opalescence with saline, and suitable controls are set up. The reaction is read after 20 minutes' incubation at 37° C., and again after 1 hour at room temperature. Pulgher believes in the utility of the method. The reaction deserves a trial on a satisfactory scale in a country like India, where an ample supply of naturally infected material is available.

Plague-like lesions and diseases met with in rats.

In the early years of plague work attention was often drawn to various plague-like bacteria that had been isolated from rats. Some of them definitely belonged to the group of coliform bacteria; they need not now present any difficulty. The Plague Research Commission in India examined thousands of *M. rattus* and *M. norvegicus*, and met with no disease in them that caused any real difficulty in diagnosis. Macalister and Brooks (1914) found some instances of infection of rats with organisms of the Gaertner group; these rats showed a characteristic condition of the liver which was not granular, but was thickly peppered with white points. They state also that trypanosomiasis, on occasion, simulated plague macroscopically; the signs were pleural effusion, congestion of the liver, congested subcutaneous tissues, and swollen lymphatic glands; Billet (1908) when working at plague in Algeria had already noted the similarity. Petrie and Macalister (1911) isolated from three *M. norvegicus* in East Anglia cultures which were very difficult to identify, either as an avirulent strain of plague bacillus or as *B. pseudotuberculosis rodentium.* Two of the rats had a granular liver and spleen, and a third a finely granular spleen. The cultures were regarded as being probably *B. pseudotuberculosis rodentium*; identification might have been rendered certain had the authors realized the value of the motility test—at that time generally disregarded—in distinguishing between these bacteria. Eastwood and Griffith (1914) isolated from 19 rats (*M. norvegicus*) cultures which during the first 2 days somewhat resembled *B. pestis*, but, unlike *B. pestis*, never formed more than a very thin and transparent layer on agar. The colonies consisted of a Gram-positive cocco-bacillus, and all the cultures were from rats which showed much pleural effusion. The description suggests that the bacterium concerned was perhaps *B. monocytogenes* of Murray, Webb and Swann (1926). Wayson (1925) has described a plague-like disease in the rats (*M. norvegicus*) in the town of Oakland in California, and the adjoining cities; the lesions resembled acute, subacute or resolving rat plague. In these rats the pleural cavities overflowed with a clear fluid. According to Wayson, the bacterium isolated was one of the Pasteurella group, but it is worth considering whether it may not have had a possible kinship with *B. monocytogenes* and with the probably identical bacterium which was found by Pirie (1927) in the gerbille (*Tatera lobengulæ*), an inhabitant of the South African veld.

Subacute and Chronic Plague in Rats.

The observations of the Plague Research Commission on resolving (chronic) plague in Indian rats (1906, 1907, 1910, 1912).

Chronic plague lesions had been produced experimentally in white rats by Kolle and Martini (1902), Hata (1904) and Douglas (Bulloch and Douglas, 1912), some years before the extensive and systematic observations on naturally infected rats that were carried out by the Commission in India (1906–12). The early workers were disposed to attribute importance to this chronic condition in maintaining the infection during the off-plague season, but the Commission concluded that chronic plague had little or no epidemiological significance. The rats that showed these lesions were rarely emaciated; and with the exception of the localized lesions, the organs were healthy. The lesions were of two kinds, 'peripheral' and 'visceral'. The peripheral lesions consisted of encapsulated abscesses corresponding in site and relative frequency to the primary plague bubo. The visceral lesions were chiefly in the spleen, either as thick-walled abscesses, usually multiple, embedded in its substance, or protruding from its surface, and often attached by adhesions to the abdominal wall, omentum or mesentery; or as greyish-yellow irregular or wedge-shaped necrotic areas in the spleen substance. Occasionally peritoneal or omental abscesses were found, but there was no evidence that these originated from an intestinal infection, because they occurred apart from demonstrable lymphatic glands; the evidence, in fact, showed that they had originated from the lesions, just described, in the spleen. Plague bacilli, when present, were not abundant in the lesions, and in most cases they were virulent; the lesions were often sterile. Most of the rats were found during and immediately after an epizootic of plague. In the village of Dhand in the Punjab, this kind of lesion was present in 28 per cent. of all the plague rats—an exceptionally high percentage. In Bombay, the incidence of the chronic lesions in *M. norvegicus* was twice as great as in *M. rattus*, corresponding to a similar incidence in rats with acute plague. There was evidence that the plague bacillus had persisted in some of the spleen abscesses for from 3 to 5 months.

A consideration of the histological findings of Ledingham (1907) in the spleen and liver of plague rats, together with their own observations, led the Commission to the conclusion that the lesions are really in process of healing and recovery. Ledingham showed that the reaction of the fixed tissue cells in the spleen may proceed to complete encapsulation of the abscesses produced by the invasion of the organ by plague bacilli, thus bringing about a more or less chronic condition. The term ' resolving ' or ' resolved ' plague was therefore preferred, since it avoided the erroneous implications in the term ' chronic plague ', and since it expressed more aptly the course of events. A careful study of the condition in the Bombay rats, *M. norvegicus* and *M. rattus*, showed that, as might be expected, no sharp line can be drawn between acute, subacute, resolving and resolved plague, and that a continuous series of pathological changes can be traced towards the final stage of healing, namely, scar tissue.

The Commission were able to produce the condition experimentally. Thus they found two instances of ' chronic ' plague out of 32 rats experimentally infected by fleas ; the lesions were a right inguinal and a right pelvic abscess. Again, in an experiment the chief object of which was to test the relative immunity of rats obtained from different parts of India, there were 1,079 survivors ; these were killed three weeks after the infective dose ; and 119, or 11 per cent., showed resolving or resolved plague lesions. The survivors of the most susceptible group yielded the largest proportion with resolving plague lesions. The lesions exactly resembled those found in naturally infected rats, and the circumstances of their occurrence clearly proved that they represented stages on the way to recovery from a moderately severe attack of plague.

In Madras, which has been free from epidemic plague, similar lesions were found ; there were 8 peripheral and 27 visceral lesions in 15,523 rats. Moreover, a certain number of young rats in Poona showed the lesions, although they could not have been exposed to plague infection. The Commission concluded, therefore, that some cause other than plague can produce lesions of this kind. The observations of Wayson on *M. norvegicus* in California are significant in this connection (see p. 191).

Finally, the Commission observed not a single rat with appearances which suggested a lighting-up of a resolving lesion into an acute infection. Such an event is unlikely to happen, because, during the process of recovery from an acute attack, rats presumably acquire a considerable immunity, and, moreover, there is no experimental evidence, either in plague or in any other similar infection, to support the idea that a chronic focus is able to kindle a fresh septicæmia.

Apart from the Indian observations little has been recorded. Typical resolving plague lesions in *M. rattus* that contained virulent bacilli were noted in Qûs, a town in Upper Egypt (Petrie and Todd, 1923). Feldmann (1908), at Kisiba in Kenya, found that the bacilli from chronic rat plague possessed a weakened virulence for rats, but a considerable virulence for guinea-pigs. In the investigation of over 100,000 rats in San Francisco, 98 per cent. of which were *M. norvegicus*, McCoy (1909^2) saw no undoubted example of the condition, and not more than 6 with lesions that strongly suggested it; this result may be associated with the high degree of immunity to plague of the rats in that city. Ashburton Thompson (1906) found no chronic plague in rats in Sydney, and discounted its importance as a bridge between epidemic periods, for the reason that he was able to trace a continuous chain of acute plague in the rats. Pirie (1927) could find no evidence for the existence of chronic plague in the veld rodents of South Africa. Williams and Kemmerer (1923) diagnosed 19 out of 89 infected rats in the New Orleans epizootic (1914–17) as 'resolving' plague (21·3 per cent.); in the second New Orleans plague outbreak (1919–22), 89 out of 584 infected rats were in the resolving stage (15 per cent.).

Plague in rats without visible lesions: rats as carriers.

Williams and Kemmerer (1923), from their experience in New Orleans, think that, especially towards the end of the epizootic, there is an increase in the number of rats, with slight lesions or with none, that yield strains which are apparently of low virulence. Bordas, Dubief and Tanon (1922) examined the rats in the recent small epidemic in Paris, and came to a similar conclusion, namely, that when the human cases ceased to appear, the number of carrier rats increased. Tanon (cited by Colombani, 1924), on the strength of these observations, seems to think that the rat acts as a reservoir of a feebly virulent strain of plague bacillus; transmits it to successive generations until it regains its activity; and thus originates a severe epizootic. Speculations of this kind have lost contact with accepted knowledge, and are scarcely credible. Pirie could find no definite evidence in favour of the hypothesis in the course of his work on the veld rodents of South Africa. Further enquiry is needed on the subject; and attempts should be made to estimate with special care, by using quantitative methods, the degree of virulence of any strains that may be isolated.

The Varying Immunity of Rats to Plague.

The Plague Research Commission (1912) tested rats from 25 towns widely scattered over India. Four places that had been free from plague yielded susceptible rats; and the results as a whole showed that the resistance of rats to plague is in general proportional to the prevalence of the disease in the locality which they inhabit; in this and similar experiments, the immunity is naturally relative to the infecting dose. A number of Bombay rats, most of which were *M. rattus*, showed a higher immunity to plague, as tested by feeding and by flea-transmission experiments, than ship-rats from Bombay harbour, which presumably had remained exempt from epizootic plague. The natural resistance of rats to plague is due, not merely to the previous reception of sublethal and immunizing doses of plague bacilli during the prevalence of the epizootic, but to the generation of a relatively immune progeny by the survivors of the epizootic. Young rats from Madras, Bombay and Poona possessed an immunity of the same order as that of the average adult rat from the same place; moreover, young rats obtained at Parel by breeding from wild rats, proved to possess the resistance of their parents. It is probable, therefore, that the process of immunity among rats does not consist in their active immunization during an epizootic and the subsequent transmission of the immunity to their offspring, but rather in the weeding-out of susceptible members. McCoy (1910) states that about 50 per cent. of the adult rats, most of them *M. norvegicus*, in San Francisco were immune, and that there was no reason for believing that the immunity was due to a previous attack of the disease. Swellengrebel and Otten (1914[1]) tested the immunity of house-rats in Java by flea-transmission experiments; the mortality figures for rats obtained from Malang and Surabaya were 74 per cent. and 64 per cent. respectively, whereas nearly all of the control guinea-pigs died. *M. norvegicus* from the plague-free port of Mobile, Alabama, when tested by the cutaneous and subcutaneous methods, yielded a considerable proportion of immune individuals (Spencer, 1922). Light has been thrown upon the mechanism of the naturally acquired immunity of Indian rats by the work of Malone, Avari and Naidu (1925), who showed that there is a definite relation between immunity or susceptibility to plague in rats and the bactericidal power of the whole blood. They regard phagocytosis as an essential part of the process, although intracellular or extracellular lysis may occur.

Ship-rats and their Relation to Plague.

M. rattus is by far the commonest species of rat on board ships. Observations made in the Hygienic Institute at Hamburg showed that in 1906, 97·2 per cent. of all rats caught on ships were *M. rattus;* the remaining rats were *M. norvegicus* (Dieudonné and Otto, 1927). The ratio of *M. rattus* to *M. norvegicus* that were captured on Australian and overseas vessels at Sydney and Melbourne in 1921–2 was 40 : 1; 5,045 rats were identified (Cumpston and McCallum, 1926). In 1913, the Hygienic Institute at Hamburg examined 10,258 rats and 173 mice from 511 ships which had arrived from plague-infected ports; 8 ships yielded in all 166 plague rats (Berlin, 1915). There are many scattered data on the incidence of rat plague in ships, but they do not appear to have been brought together in a convenient form.

PLAGUE IN ANIMALS OTHER THAN RATS.

Mice, Cats, Domestic Animals, &c.

The mouse is not an important carrier of plague. In the San Francisco outbreak it was considered to be fairly harmless, for the reason that it does not migrate much; and in Australia its insignificant flea-infestation has caused it to be acquitted of complicity in the spread of epizootic and epidemic plague. The Commission in India proved that mice were relatively immune, whether they came from Bombay, an infected city, or from Madras, which has remained free from plague.

Cats have often been reported as dying from plague, and for this reason they should not be used for hunting rats during an epidemic.

The musk-rat, an insectivorous animal, inhabits houses and is sometimes mentioned in accounts of plague outbreaks. The Indian and Javan species (*Crocidura cærulea*) is highly resistant to the infection. Hirst (1926, 1927) thinks it an advantage to return *Pachyura cærulea*, the musk-rat of Colombo, to the houses when trapped, because it is immune to plague, and because it is hostile to the domestic rat. According to Leger and Baury (1923[3]), the African shrew-mouse (*Crocidura stampflii* Jentink) in the town of Dakar in Senegal, is infected to the same or an even greater degree than the rodents. Plague infection was found in the shrew-mouse (*Crocidura murina*) of Cambodia by Mathis (1923), and previously by Kérandel (1915).

The following animals while living in domesticated conditions have been observed to be naturally infected: ferrets, laboratory guinea-pigs and rabbits, monkeys in the Zoological Gardens in Bombay, and marsupials in the Zoological Gardens in Sydney.

The Animal Carriers in the Principal Foci of Plague in the World.

Java.

Three sub-species of *M. rattus* are met with: (1) the house-rat (*M. griseiventer* Bonhote), (2) the small house-rat (*M. concolor* Blyth), and (3) the field-rat (*M. diardii* Jentink). *M. concolor* usually nests outside houses, but when the ordinary house-rat is exterminated by plague it takes its place, and is a more dangerous carrier because its small size allows it to make its way along the hollow bamboo timbers more easily than the other species, and, therefore, it invades the houses in larger numbers (Flu, 1924). Both *M. norvegicus* and *M. rattus* infest the coastal towns, for example, Surabaya. The two varieties of house-rat carry plague; the field-rat is of little or no importance as a plague carrier, partly because its flea-infestation is very low.

X. cheopis is the important species of rat-flea for plague in Java; *Pygiopsylla ahalæ*, the flea of the field-rat, even when it is hungry does not bite man, and is not so good a transmitter of plague from rat to rat as *X. cheopis* (Swellengrebel and Otten, 1914[1]). The flea average of rats in Java is in general low.

Egypt.

In Upper Egypt, *M. rattus* is the most important plague rodent, and *X. cheopis* is the principal flea (90 to 100 per cent.) on the species of rodents associated with man in human habitations. The Cairo spiny mouse (*Acomys cahirinus*) is quite common in houses in Cairo and Upper Egypt, but on account of its low flea-infestation it is not an important plague carrier. *M. norvegicus* is more widely distributed as a house-rat in the Delta than in Upper Egypt; and it was trapped in greater numbers than *M. rattus* in the feluccas which ply on the Nile between Cairo and Aswân.

The common field-rat, *Arvicanthis niloticus*, carries numerous *X. cheopis*, and occasionally inhabits houses on the outskirts of towns and villages, but it has not been found to be plague-infected (Petrie and Todd, 1923).

Russia.

Within recent years research into epizootic and epidemic plague in the steppe-region of Southern Russia, a well-known endemic focus, has been actively pursued under the guidance of Zabolotny and of Nikanorow; the insect parasites have been studied by Wagner, the well-known authority. The spermophile, a burrowing rodent which hibernates in the winter, is the important carrier of plague: there are three species: (1) *Citellus suslika*, (2) *C. musicus*, and (3) *C. mugosaricus*. Klodnitzky (1926) states that in this region, the highly susceptible yellow spermophile (*Cynomys fulvus*) is the chief source of the infection. The steppes on the left bank of the Volga are densely populated by *C. mugosaricus*, and the sandy areas by *Cynomys fulvus*; the northern area on the right bank is inhabited by *C. guttatus*; and the area towards the south by *C. musicus* (Lebedev, 1926). The fleas that infest these rodents are: (1) *Ceratophyllus tesquorum* Wagn., (2) *Ctenophthalmus pollex*, (3) *Ctenophthalmus breviatus*, and (4) *Neopsylla setosa* Wagn.; the first and the last mentioned are able to bite man. The maximum prevalence of fleas is in the autumn, with an average of 10·8 for September. Golov and Ioff (1925) do not doubt the importance of the part taken by fleas in the spread of epizootics among spermophiles. They found that the fleas when experimentally infected, kept thereafter at from 7 to 10° C. with an air humidity of 80 to 92 per cent., and fed on a fresh spermophile every other day, carried plague bacilli for more than two and a half months. They observed the appearances of obstruction and regurgitation, as described by Bacot and Martin. Gaisky (1926) inoculated hibernating spermophiles with plague cultures, and found that the infection remained latent in some of them for periods extending to 138 days. Three of them passed the winter in the hibernating state, and died of plague at the time of the natural awakening; the bacilli were found mostly at the site of inoculation. Similar results had previously been obtained by Dujardin-Beaumetz and Mosny (1912), who used in their experiments a species of marmot, *Arctomys marmota* Schreb., the Alpine marmot.

Camels, which are used as beasts of burden in the steppe-region of Southern Russia, have been under suspicion as plague carriers for a good many years. Nikanorov (1926) has investigated their susceptibility to plague bacilli, when administered by various routes. An experimental infection is not easily induced, but infection may follow when the resistance is weakened by over-work or from other causes. This author believes that the infective fæces of spermophiles contaminate the forage eaten by camels; that hay may be infected by plague-infected mice; and that camels may receive the infection in this way and so initiate human outbreaks. His suggestions conflict so sharply with experience gained elsewhere, under both natural and experimental conditions, that it seems best to await further information.

A contribution by Nikanorov (1928) in a recent publication on rodents and their fleas gives the most useful review of the epidemiology of plague in South-east Russia that has yet appeared.

Transbaikalia.

This region, together with extensive tracts of Northern Manchuria and North-east Mongolia that are conterminous with it, has long been known as an endemic focus in which outbreaks of bubonic and pneumonic plague have occurred ; and it has been the source of the recent widespread epidemics of pneumonic plague in Manchuria and Middle China. That the tarbagan is the reservoir and origin of the infection admits now of no doubt. Little is as yet known of tarbagan plague and the mode of its transmission in nature. Guinea-pigs have been infected by the agency of tarbagan fleas (*Ceratophyllus silantievi* Wagn.), but there is no record of flea-transmission experiments with tarbagans. How exactly the infection persists in the burrows of the tarbagan throughout the severe winters has not yet been ascertained, although the collateral evidence points to its being carried over in the tarbagan flea. As this rodent is esteemed as an article of food, and as the fur is a valuable commodity, direct infection when skinning the animal must not infrequently take place, just as it does with the spermophile in Russia and the ground-squirrel in California ; in all these places human bubonic plague is characterized by a high proportion of axillary buboes. The tarbagan flea is able to bite man and imbibe his blood.

South Africa.

From the first appearance of plague in South Africa in February, 1899, until the year 1914, human plague was associated with rat plague. The problem assumed a different aspect when in August, 1914, a small outbreak of pneumonic plague appeared in Cape Colony on a farm about 140 miles from Port Elizabeth. Although most of the succeeding cases on the estate were bubonic, signs of an accompanying rodent infection were not manifest. Scattered outbreaks on farms continued to occur throughout the Union in circumstances that gave no clue to the source of the infection, but in 1920 Mitchell and Haydon ascertained that the distribution of the outbreaks corresponded roughly with the sandy stretches of veld frequented by gerbilles (*Tatera lobengulæ*). In February, 1921, an epizootic in gerbilles and multimammate mice was responsible for a fatal case of human plague near Bothaville ; this outbreak gave the first definite proof that incriminated veld rodents. Natural plague has since been found in 11 species of veld rodents and in the yellow mongoose. There is evidence that rodent infection on the veld is spread by contiguity from rat infection in the coastal region. Thus the existence of plague among the veld rodents was recognized for the first time in October, 1903, at Knysna, on the coast of Cape Colony, when the striped mice

(*Rhabdomys pumilio*) inhabiting the dense woods near the town, were shown to be suffering severely from plague; the origin of the infection was an outbreak of plague among the rats in the town. The infection probably travelled through the coastal forest belt among the striped mice, and was passed on by them to the gerbille colonies, and to other associated veld rodents, in the farming country farther inland. The epidemiology of human plague on the veld is bound up with the bionomics of the veld rodents and of their carnivore associates, the suricate and the yellow mongoose.

The evidence points to the predominating role of the flea as the transmitting agent in the rodent epizootics. The flea, too, is an all-important agent in the persistence of plague in the rodent population, and experiments that were carried out in rodent-proof and flea-proof runs on the veld, with gerbilles as the experimental animal, showed that in the absence of fleas plague did not persist in the burrows even for one month; and that when fleas were present, the infection persisted abundantly for two months, and to a lesser extent even for three months. These results explain how plague is carried over from one summer to another, and over periods when the rodent population is scanty (Pirie, 1927).

Four of the species of fleas that infest the veld rodents can imbibe human blood, namely, *Dinopsyllus lypusus* J. and R., *Xenopsylla eridos* Rothsc., *Xenopsylla erilli* Rothsc., and *Chiastopsylla rossi* Watrst. Except *X. erilli*, these species may transmit plague to rodents under experimental conditions.

A comprehensive account of the history of plague in South Africa during the past 30 years will be found in the report by Mitchell, Pirie and Ingram (1927).

California.

Natural plague infection among ground-squirrels (*Otospermophilus beecheyi* Richardson) was discovered in the year 1908; this rodent was first regarded as suspect in 1903, and in 1904 its susceptibility to experimental plague was proved. The features of the natural disease are those of a subacute infection, and there are indications that the strain of bacillus is somewhat less virulent than strains from other species of animals. Thus, of 246 plague squirrels the lesions of 60 were those of acute plague; of 152, subacute plague; and of 34, residual plague, that is, resolving plague buboes. The post-mortem appearances and particularly the purulent and caseous foci in the spleen, liver and lungs resemble a subacute form of plague in which there has been time for reactive tissue changes; a primary bubo is often seen.

Guinea-pigs inoculated cutaneously with material from rat plague died on the average about one day earlier than those inoculated with material from squirrel plague. Rupert Blue thinks that human plague derived from squirrels is apt to be less virulent than that derived from rats. This opinion seems to be based on the case of a boy, 13 years old, who died on the sixteenth day of his illness from an attack of plague which was characterized by an axillary bubo, a pustular eruption on the tenth day of illness, and numerous irregular nodules in the lungs (McCoy and Wherry, 1909). Similar pyæmic cases associated with rat plague have, however, been described by Seyfarth (1925) and others.

Squirrel plague does not seem to be more prevalent at one season than another. Healthy squirrels from localities where plague has prevailed for several years are more resistant to artificial infection than those from plague-free places.

The flea most often found on ground-squirrels in California is *Ceratophyllus acutus* Baker ; *Hoplopsyllus anomalus* Baker, is also found. The American workers have shown beyond doubt that plague may be conveyed from one ground-squirrel to another by means of *C. acutus.*

Detailed information on plague in the ground-squirrels of California is contained in a series of papers by McCoy and his colleagues (McCoy, 1909[3], 1911 ; McCoy and Wherry, 1909 ; McCoy and Chapin, 1912).

Bubonic Plague in Man.

THE PATHOLOGY OF BUBONIC PLAGUE.

The Significance of the Terms ' Bubonic ' and ' Septicæmic ' Plague.

The terms ' bubonic plague ' and ' septicæmic plague ' are in common use, but are not precise enough to be really satisfactory ; they indicate that in the one form of the disease an obvious primary bubo is present, and that in the other no recognizable primary bubo can be found. Bubonic plague is, however, often accompanied by a septicæmia, and, on the other hand, an inflammatory enlargement of the deep lymphatic glands is present in septicæmic plague. Indeed, Crowell (1915), in his studies of the morbid anatomy of bubonic plague, recognizes only a ' primary bubonic ' and a ' primary pneumonic ' form of plague. He classifies the ' bubonic ' group into (1) uncomplicated cases, (2) bubonic plague with early septicæmia or without superficial buboes, (3) bubonic plague with secondary pneumonia, (4) bubonic plague with secondary meningitis, and (5) bubonic plague with secondary cutaneous lesions. The degree of resistance offered by the tissues of the patient to the bacillary invasion probably determines the extent of the reactive changes in the lymphatic filter and the rapidity with which the blood stream is invaded ; these morbid processes have no relation to the virulence of the infecting strain, since freshly isolated human strains possess a uniformly high virulence.

The Portal of Entry of the Bacillus.

The usual mode of infection is through the skin by the bite of the infected rat-flea, but direct infection may occur from accidents in the laboratory or post-mortem room, and by handling or skinning infected animals. A local reaction, in the form of a pustule at the site of infection, is rarely seen ; a lymphangitis spreading towards the primary bubo is also a rare occurrence. A few cases are on record of a primary conjunctivitis following direct infection with sputum from a patient suffering from a plague pneumonia. The tonsil seems occasionally to be the site of entry of the bacillus. The intestinal mucous membrane is not now regarded as a portal of entry.

The Lesions in Bubonic Plague.

The Austrian Commission (1898) distinguished between a primary bubo at the usual sites—inguinal, axillary, and cervical—and an associated primary bubo affecting the nearest proximal group of glands ; they named the first a primary bubo of the first order, and the second a primary bubo of the second order. They gave the name of secondary bubo to those glands which are infected by way of the blood stream in septicæmic plague. The widespread hæmorrhages and degenerative changes in the tissues are due to the generalized absorption of the specific bacillary toxin ; and the foci of necrosis associated with bacillary emboli in the organs are doubtless also to be ascribed to the local liberation of the endotoxin. The high concentration of toxin within and near the primary bubo falls with special severity upon the larger veins in its neighbourhood, and accounts for the hæmorrhagic infiltration of their walls and the consequent passage of bacilli into the blood stream.

The Bacteriæmia in Bubonic Plague.

The Plague Research Commission (1906, 1908) made two series of observations. In the first series of 74 samples from 28 patients, of whom 5 recovered, 30 were positive ; in only 6 of the 30 samples were plague bacilli seen microscopically. The number of bacilli per c.cm. of blood was not high in comparison with the number found in septicæmic rats ; the highest number was greater than 1,000,000 in a sample taken 20 hours before death ; in this sample fairly numerous bacilli were seen microscopically. In another patient there were more than 10,000 bacilli per c.cm. 81 hours before death ; and in a patient in the second series bacilli were present in the blood from 12 to 17 days before death. The bacteriæmia usually increases until death takes place, but it may decrease or show fluctuations. The shorter the interval before death the greater is the chance of recognizing the bacilli in the blood microscopically. Two patients who gave a positive blood-culture recovered ; in one of them, 1 c.cm. yielded 10 colonies, and in the other there were 500 to 600 colonies per c.cm. two days after the reputed date of attack.

The results published by different workers are not exactly comparable, because they depend on the amount of blood taken ; naturally, the larger the amount the more likely is the culture to be positive.

The Presence of Plague Bacilli in the Urine and Fæces.

The Plague Research Commission in India (1908) examined the urine from 27 patients, and found a plague bacilluria in 8 of them (30 per cent.).

From subcutaneous tests on guinea-pigs it appeared that when the urine is infective there is usually a severe septicæmia at the time of examination; on the other hand, the blood may contain many bacilli and yet the urine may not be infective. The escape of bacilli into the urine is related to the degree of degenerative change in the kidneys. In another series, 57 samples of urine obtained from 37 patients were examined, and 11 of the samples (19·3 per cent.), when inoculated subcutaneously, killed a guinea-pig. The Commission never succeeded in infecting guinea-pigs with urine by the cutaneous method. They examined the fæces of 20 patients by means of the cutaneous method in the guinea-pig. Only once did a guinea-pig die, and this result may have been due to accidental contamination with blood derived from a rectal abrasion while taking the sample.

THE CARRIER STATE IN HUMAN BUBONIC PLAGUE.

In rare instances the bacilli persist for a considerable time after an acute attack of plague, as for example, in the primary bubo; a patient in this condition may be able to walk about, and eventually he may recover completely. Uriarte (1924) describes an instance of bubonic plague with iridocyclitis and hypopyon as a rare complication. After the patient had received serum treatment an apparent cure resulted, but three weeks later he died of a plague meningitis.

A number of papers have appeared within recent years, chiefly in French journals, on the persistence of virulent plague bacilli in small enlarged glands, which are sometimes painless, and which cause at the most comparatively slight symptoms (Leger and Baury, 1922 and 1923[4]; Tanon and Cambéssèdes, 1923; Uriarte, 1924; Durand and Conseil, 1927; Boston, 1927). For the successful demonstration of the bacilli, Uriarte (1925) recommends excision rather than puncture of the gland. Durand and Conseil have suggested that if carriers of this kind are attacked by influenza or measles, their resistance will be lowered, and a bacteriæmia may supervene with a possible spread of the infection by means of plague-carrying fleas or by a respiratory complication. In a recent review of the subject, Nikanorov (1927) goes so far as to say that plague bacilli may be found in the blood of convalescent patients, and even in persons who are in good health; that the presence of virulent plague bacilli in healthy persons has been confirmed; and that healthy carriers act as a source of infection to members of their family, apparently by means of ectoparasites. He thinks that the appearance of plague in the absence of an epizootic may be explained in this way. These statements suggest a too liberal interpretation of work in which there are still many gaps; further observations, both bacteriological and epidemiological, are needed to assess the importance of the carrier state in plague.

NATURAL AND ACQUIRED IMMUNITY TO PLAGUE.

Dieudonné and Otto (1927) take the view that all persons are susceptible, and that apparent differences in susceptibility are due to varying circumstances in the mode of life. In South Africa, Europeans show the greatest resistance to the infection, as manifested by a high proportion of mild cases among them, and a comparatively low death-rate. Asiatics are said to show the least resistance, and to be specially liable to the pneumonic and the septicæmic forms (Mitchell, Pirie and Ingram, 1927).

Considerable protection is conferred by an attack of plague, but second attacks sometimes occur.

THE DIAGNOSIS OF BUBONIC PLAGUE.

Diagnosis during Life.

The ordinary methods for isolating the bacillus should be utilized. Kolle and Otto (Dieudonné and Otto, 1927) recommend the method of stabbing the root of a rat's tail with a hollow needle containing the suspected material, because, if introduced subcutaneously, pathogenic rat bacteria other than *B. pestis* may kill the rat. A suspicious bubo should be punctured in the region of the periglandular tissues, where the bacilli are most numerous and, as Flu (1924) points out, a fine hollow needle should be used, and care taken to avoid unnecessary pain, since this may cause shock in a patient whose heart has been poisoned by the endotoxin of the plague bacillus. The microscopical examination of the blood is too seldom positive to afford help ; blood-culture is a much safer guide, and the blood itself may be inoculated into animals.

The identification of suspected cultures by means of agglutination with a specific plague serum.

Plague serum is strongly specific, except for its agglutinating action on the closely related *B. pseudotuberculosis rodentium.* The titre of a good serum is a dilution of about 1 in 6,000. Controls with normal horse-serum, and with a known plague culture are advisable. It is not easy to get a uniform suspension of plague bacilli in saline, and this seems to be due to the slimy character of the cultures. The temperature at which the culture is grown is thought to affect the character of the suspension ; according to Pulgher (1922) it should not be higher than 23° C., and a rather dry agar slope should be used.

d'Aunoy (1923) grew cultures on agar for from 50 to 60 hours, washed off the growth, filtered the suspension through sand, and made a final suspension in saline at pH 6·8. He states that suspensions thus prepared, exhibited no spontaneous agglutination over long periods when kept at from 10 to 38° C. Pirie (1927) used from 0·1 to 0·2 per cent. saline instead of normal saline for his suspensions, and 0·1 per cent. formalin for killing and preserving them. The bacilli were washed off agar plates which had been incubated at from 30 to 37° C.

The question of the existence of serological types. Hetsch and Rimpau (Kolle, Hetsch and Otto, 1904) tested numerous strains from various sources, and observed no difference between multivalent and univalent sera in their protective power for rats. They, therefore, concluded that *B. pestis* possesses a receptor apparatus of remarkable uniformity: a statement which agrees with the agglutination experiments carried out by Otto from the same point of view. Other members of the Pasteurella group exhibit no such uniformity of antigenic structure. These results render it unlikely that *B. pestis* will prove to be separable into diverse serological types, and at the same time they increase confidence in the interpretation of agglutination tests with suspected cultures.

The serological diagnosis of plague.

Serological reactions, as might be expected, cannot be relied upon for the diagnosis of an acute disease like plague. The agglutination reaction is apt to be weak, and a negative result has no significance. The complement-fixation reaction has been used by Joltrain (1920) and Simard (1921), and doubtless it gives useful information in convalescent cases of suspected plague.

Diagnosis after Death.

In tropical countries, where, on account of popular prejudice, routine post-mortem examinations cannot be carried out, recourse must be had to puncture of the organs. In Java, spleen puncture is considered to be a valuable method. The French Colonial Medical Officers have used liver puncture a good deal. The value of methods of this kind is directly proportional to the interval between the time of death and the examination, because if putrefaction has begun, intestinal bacteria which have invaded the liver and spleen may simulate the plague bacillus microscopically; a culture should always be made when possible. Pons (1926) gives some evidence for thinking that in experimental animal plague and in human plague the bone-marrow may contain pure plague bacilli 20 hours after death, although the organs yield mixed cultures.

THE TREATMENT OF BUBONIC PLAGUE.

Serum Therapy.

The Plague Research Commission in India (1912) undertook an examination of the therapeutic results of antiplague serum. The conclusion to which they came was guarded, and indicated that no appreciable benefit was conferred by its use. Later experience, however, appears to be favourable to this form of treatment, when the serum is given early in large doses intravenously. Some of the accounts speak of an amelioration of the symptoms due to an antitoxic action of the serum (Barrett Heggs, 1923, and others). As already stated, the problem of specific serum therapy in plague is likely to remain a subject of controversy until research has determined the essential elements in the serum, whether antibacterial or antitoxic, and how they may be measured. A review of the clinical records gives encouragement for such a quest. The opinion of McCoy and Chapin (1920), that in plague outbreaks energy should be diverted from the use of plague sera and vaccines to measures directed against rodents, may be applicable to well-organized communities, but not to countries like India, where the difficulties of plague prevention on strictly logical lines are immense, and where the treatment of patients is an urgent matter.

The Use of a Plague Vaccine as a Curative Agent.

Row (1913) and Nikanorov (1924) think they have relieved the symptoms of bubonic plague by injecting a vaccine prepared by killing the bacilli in glycerin without the use of heat or an antiseptic, but confirmation of their results is needed.

Chemotherapy.

There is no definite information under this head. Some observers have used preparations of iodine intravenously. Mercurochrome, when given experimentally to susceptible animals, is without effect on the course of the infection.

Treatment by Bacteriophage.

This form of treatment has been tried in India by d'Herelle, and by Naidu and his colleagues, but has not so far proved its efficacy (Mackie, 1928).

PROPHYLAXIS AGAINST BUBONIC PLAGUE.

Vaccination against Bubonic Plague in Man.

There has been much discussion on the utility of plague vaccines as a preventive measure against plague. It is difficult to submit the published data to an exact statistical analysis, because of the impossibility of estimating the degree of exposure to infection of the persons in the inoculated and the uninoculated groups. Plague is essentially an infection attaching to localities, not to persons, and even in infected houses the rule is that only one person is attacked. In India, where vaccination has been practised on a very large scale, there is a general belief in its utility, and apparently with good reason (Table VII), but, on the other hand, plague workers in Java have been unable to note any protective action after the use of Haffkine's vaccine and the German Commission's vaccine. Recent experience in Greece with agar saline emulsions killed at 65° C. —a higher temperature than seems advisable—did not support its use, for some of the vaccinated persons contracted plague, and the clinical course and mortality rate were the same whether the patients had been inoculated or not (Seyfarth, 1925). An analysis of the figures for vaccination on a much larger scale in Baghdad, from 1919 to 1923 inclusive, shows that a vaccine definitely protects both against attack and death, a conclusion which supports Indian data of the same kind (Barrett Heggs, 1923). McCoy and Chapin (1920), in a critical review, state their belief that there is no evidence that vaccination has ever controlled an outbreak. The present writer, however, has obtained evidence that epizootics of *B. pseudotuberculosis rodentium* in guinea-pigs can be controlled by a specific vaccine; in an outbreak among the stock of guinea-pigs at the

Lister Institute, Elstree, vaccination seemed to have a dramatic effect in causing a rapid disappearance of the infection. Dr. C. Todd had a similar experience in the same laboratories. More work on the preparation of plague vaccines is necessary, in order to increase their immunizing efficiency, and at the same time to reduce the local and general reactions which are apt to follow their administration. One advantage of an improved vaccine would be that a series of doses might be accepted, with the production of a solid immunity. Flu (1925) obtained exceptionally good results in guinea-pigs when he immunized them with three serial doses of an unheated, carbolized, spun extract of plague bacilli. The use of a living avirulent vaccine has been advocated by Strong (1912). This is regarded at the present time as a method which is not free from danger, and yet it deserves to be further investigated in view of the fact that a living vaccine is more potent than a killed vaccine.

TABLE VII.

Data indicating the efficacy of plague vaccination in India, abstracted from the Report of the Haffkine Institute for the year 1927.

	Population at risk.	Number of plague cases.	Number of plague deaths.	Percentage of survivors from attacks.
Uninoculated ..	3,227,842	59,668 (1·84%)	50,735 (1·5%)	14·9
Inoculated ..	1,937,213	4,126 (0·21%)	1,620 (0·08%)	60·7

Vaccination per os *in man.* This has been tried on a small scale; experimental evidence scarcely supports it use.

Serum Prophylaxis against Bubonic Plague in Man.

The immunity conferred by this means lasts for 2 or 3 weeks only.

Combined Immunization with Vaccine and Serum: the Simultaneous Method.

This method has been recommended by Shiga (Kraus and Levaditi's Handbuch, 1908) and by other workers, but it has not become general. Where exposure to infection has been recent, active immunity may be produced under cover of the more rapid but transient passive immunity conferred by the serum.

THE PREVENTION OF PLAGUE.

The Scope of the Problem.

Ever since the present pandemic of plague spread in 1894 from Hong Kong, the problem of its prevention has engaged the attention of sanitarians in nearly every country in the world. An idea of the scope of the problem is given in the statement that between 1896 and 1925 11 million persons died of plague in India, and that in 1923 more than 250,000 cases of plague were reported throughout the whole world. Even Europe has not been exempt in recent years: in 1919–20 there were about 120 cases in Paris and Marseilles, and from the 1st of January to the 1st of November, 1924, there were 215 cases in Greece.

The Nature of the Problem: how Plague is Spread.

Since plague is carried by the rat and the rat-flea, preventive measures must aim at the extermination of the rat. This ideal is impossible of attainment at the present time, but attempts may be made to restrict the activities of the transmitting agents so as to prevent their spread within infected areas and into hitherto uninfected places. Plague rats with their fleas may make their escape from a ship on its arrival at a port, or they may be transported to warehouses as stowaways in goods, or be carried by rail to inland localities. Thus, Heiser (1913) regarded the outbreak of plague in 1912 in Manila as due to rats imported in cargo from infected centres in China and Japan, because no infected ship-rats were found; the rats at the water-front were free from plague, and, indeed, the wharves were free from rats and practically rat-proof. Rats could easily, he thought, find refuge in baskets of foodstuffs or in crates of goods packed in straw. According to Flu's observations (cited by Hirst, 1926, 1927), the commonest and most important medium of importation of the infection is the plague-infected rat-flea in cargo, such as rice and gunny bags. Hirst is of the same opinion. In the years 1901 to 1905 plague spread to various localities in Cape Colony, apparently by the railway routes; it was probably conveyed by rats and their fleas which infested forage or crated goods brought from the infected ports in the Colony. During the twelve months ending 30th June, 1925, 991 rodents, all of them *M. rattus*, with the exception of a few house mice, were found in railway trucks on their arrival at the goods station at Johannesburg. During the next year, 378 *M. rattus* and 3 house mice were found in 330 trucks; the trucks loaded with maize or grain had the most rodents; all the rodents were free from plague. The excursions of *M. rattus*, apart from passive transport, are very restricted; the belief that in Java this species of rat spreads the infection by travelling from village to village has been shown to be erroneous (Flu, 1924). The Plague Research Commission in India (1907) brought forward evidence for the view that the disease within infected localities in India is spread by the transport of infected rat-fleas in the clothing or in the effects of persons who move from place to place. The workers in Java think that this mode of dispersal is not an important one in that country, but it should be remembered that the rats in India are much more heavily infested with fleas than those in Java, and that the conditions for the spread of the infection in India appear to be specially favourable. Flu (1924) thinks that railway trucks, ox wagons, and hill ponies with packs are the common means in Java for the conveyance of infected rats and their fleas, and that personal baggage is less important.

Endemic Centres of Plague.

There are several endemic centres of plague which favour the persistence and the spread of the disease ; most of them are in remote parts of Asia, and none have been thoroughly investigated. Certain features are common to all. They are situated in mountainous regions, and are inhabited by primitive peoples who live in grossly insanitary conditions in close association with their domestic animals, a state of affairs which is ideal for rat infestation. The climatic conditions—a fairly warm summer and autumn, and a cold winter—are favourable for the persistence of plague. At the present time, centres of plague which are associated with a widespread infection of burrowing rodents have assumed great importance as endemic areas ; they present a problem of extreme difficulty.

Methods for the Reduction or the Extermination of the Rat Population.

Systematic rat trapping is useful. A form of cage trap is the best if it is properly baited and kept in order. Kunhardt (1919) is of the opinion that barium carbonate mixed with dough is the best poison bait. Cats and dogs should not be employed as rat hunters, because although in some measure resistant to plague, they may carry about infected rat-fleas. The bacillary viruses are of doubtful value. French workers have used chloropicrine as a killing agent for rats and their fleas (Bouffard, 1924).

Graham (1925) has given an interesting account of an intensive rat campaign by trapping in Delhi in 1923 and 1924. Although this city is open on all sides to plague infection, the transport of corn and the traffic with the badly infected surrounding regions were not restricted. An attempt was made to gain the confidence of the people by means of lectures and propaganda. As a result, there appeared to be a noticeable reduction in the death-rate from plague. Norman White (1918) has pointed out the danger arising from the accumulation of grain in warehouses that are inadequately protected against rats. Graham, too, stresses the necessity for constructing rat-proof grain warehouses, and for abolishing the custom of piling up grain in the native houses. Paterson (1924) reports an excellent result in rat destruction achieved by a native tribe in Kenya, the stimulus to effort being the loss of grain caused by rats, and the frequent repetition of the story of the rat-flea.

The problem of rat extermination throughout a large province or district has been discussed by Kunhardt and Chitre (1920–1[1 & 2]) on the basis of their experience in India. They think that a temporary reduction in the rat population just before or during the off-plague season should be aimed at in the comparatively few places which threaten to carry the infection over that period. Such places are the large villages and the towns, and those villages which are infected late in the plague season, in other words, places in which the conditions enable a sufficient number of plague rats to keep the infection smouldering during the non-epidemic season.

Kunhardt and Chitre believe that, when the conditions again become favourable, these off-season foci are more effective in spreading the disease than infection introduced from outside.

House improvement schemes have been organized on a considerable scale in Java. Wherever it is possible this is one of the best policies that can be adopted to combat plague, because rats are thereby denied the opportunity of living in close association with man.

Much has been written on measures for dealing with infected ships by means of such substances as sulphurous acid gas, generator gas, and hydrocyanic acid gas. Creel and Simpson (1917) compared the effects of sulphurous acid gas with those of hydrocyanic acid on ships at New Orleans by noting the number of rats killed by the fumigant and the number of rats trapped after fumigation ; they believe that hydrocyanic acid is far more effective in the fumigation of loaded holds than sulphurous acid gas. The preventive aspect of ship plague has been less studied. By means of rat-trapping, Grubbs and Holsendorf (1925) made an intensive and prolonged examination of a large passenger vessel, and observed how, in the course of installing rat-proofing devices, the number of rats that were caught greatly diminished. They believe that if shipbuilders adopted rat-proof construction, the additional cost would not be considerable. The authors give details of the methods that may be used, and of the principles underlying them. It is obvious that co-operation between the Port Medical Officer, the Ship Medical Officer and the naval architect in this matter would lead to better results, not only by abolishing shelter for rats, but by facilitating the fumigation of vessels. Measures of this kind, to be generally effective, must clearly be the subject of international agreement.

Primary Pneumonic Plague.

THE PATHOGENESIS OF PNEUMONIC PLAGUE.

The relation of primary pneumonic plague to other forms is shown in the following scheme :

- The infected rodent-flea
  - Rodent plague.
  - Human bubonic plague with a secondary plague pneumonia.
    - Primary pneumonic plague.

The Pathogenesis of Secondary Plague Pneumonia.

Most authors are now agreed that primary pneumonic plague originates as a rule from a person suffering from bubonic plague in whom a secondary plague pneumonia has supervened. Crowell (1915) found three types of pulmonary lesion in bubonic plague, (1) the ordinary broncho-pneumonic form which is associated with the aspiration of infective particles; (2) true peripheral infarcts; and (3) few or many widespread nodules of metastatic origin in the lung. Apart from these focal lesions, varying degrees of congestion and œdema of the lungs were frequently seen. In Crowell's view lesions in the tonsils, in the cervical lymphatic glands, and in the lungs show no constant correlation. Strong, Crowell and Teague (1912) refer to the experience of Albrecht and Ghon (1898) that in Bombay, in 44 cases of acute plague, foci in the lungs were observed 9 times; they comprised 3 primary plague pneumonias, 4 metastatic or secondary plague pneumonias, and 2 aspiration pneumonias from infective material derived from the tonsils; numerous plague bacilli were present in all the lesions. Flu (1924) states that the lungs in bubonic plague, when examined *post mortem*, are affected in at least 25 per cent., and that a secondary plague pneumonia can be diagnosed during life in 5 to 8 per cent. of patients who are suffering from bubonic plague. The German Commission (1899) noted the frequent occurrence of lesions in the lung in patients with cervical or axillary buboes. Crowell's observations (1915) on the morbid anatomy of plague seem to confirm the belief that, when the primary bubo is situated in the axilla, the deep cervical glands tend to become primary buboes of the second order, and to initiate an infection which spreads to the lung on the same side. Seyfarth (1925) also points out that the inflammation often extends to the pleura from an axillary bubo, and that lung complications more frequently occur when the primary bubo is in the axilla or neck.

The Origin of Primary Pneumonic Plague from a Secondary Plague Pneumonia.

In his summary of the subject, Wu (1926) admits the difficulty of knowing exactly how pneumonic plague epidemics arise, and states his belief that the chief factor is the extent of the secondary lesions in the lung of the patient who originates the outbreak, and not the frequency of occurrence of these lesions during the course of the bubonic epidemic. It is reasonable to attach importance to both factors. Heckenroth (cited by Wu, 1926) has suggested that the slighter infectivity of secondary plague pneumonia, as compared with the primary form, is due to the attenuated virulence of the bacilli brought about by the antibodies produced in the primary bubo. Reflection on this statement makes it apparent that, in a patient with a secondary plague pneumonia, the co-existing immunity must limit the extent of the pneumonic process, in contrast with the widespread invasion of the lungs which follows the aspiration of infective particles in a person who contracts primary pneumonic plague, since at the time of infection he possess no immunity.

Other Possible Modes of Origin of Primary Pneumonic Plague.

In severe septicæmic cases associated with flea-borne infection, the bacilli are said to be especially numerous in the fluid from the œdematous lungs, when the sputum is examined shortly before death. Hirst (1926, 1927) observed that, about the time of a small outbreak of seven cases of pneumonic plague in Colombo, the bacilli were exceptionally numerous in the lungs of persons dead of plague septicæmia. The expulsive efforts, while coughing, of the patient who is suffering from a septicæmia are likely to be feeble, and this circumstance doubtless lessens the risk of transmitting the infection to persons in contact with him. The Plague Research Commission (1908) commenting on the case of a septicæmic patient whose urine killed a guinea-pig when administered in a subcutaneous dose of 0·0001 c.cm., remarked that attendants on such a patient are exposed to the risk of contracting primary pneumonic plague from the spraying of bacilli in the air while he is micturating.

A Co-existent Infection as a Predisposing Factor in the Origin of Epidemics of Primary Pneumonic Plague.

Plague workers have long been puzzled to account for the haphazard outbreaks, on a small or large scale, of pneumonic plague. Speculations have naturally arisen in attempts to solve the problem. It is certainly remarkable that India, with an enormous mortality from bubonic plague, has been virtually free from pulmonary plague, whereas the pneumonic plague epidemic in the winter of 1903–4 in Kashmir with 1,370 cases, and the three extensive epidemics since the winter of 1910–11 in Manchuria and Middle China with a total of nearly 75,000 deaths, were associated at their commencement with insignificant outbreaks of bubonic plague. A recent suggestion by Norman White (1923), that the plague bacillus alone cannot cause widespread epidemics of pneumonic plague, and that a symbiosis of *B. pestis* with some other micro-organism is an essential factor, has received both support and criticism. He regards the plague bacillus as the chief lethal partner in this hypothetical association. Nicolle and Gobert (1924) think that the small pneumonic foci which they have observed in Tunis can be explained on this hypothesis, and they regard the virus of influenza as the accompanying germ. Their hypothesis presupposes two further conditions, (1) the existence of healthy human carriers of plague bacilli, and (2) the coincidence of epidemics of pneumonic plague and of influenza. They refer to the observations of Leger and Baury (1922) at Dakar, where 3 healthy natives out of 10 were found to harbour virulent plague bacilli in indolent buboes in the groin. Nicolle and Gobert suppose that if influenza attacked plague carriers of this type, the consequent lowering of their resistance would favour dissemination of the plague bacilli, with a special incidence upon the lungs, analogous to that which occurs in influenza. They state that in Southern Tunis there have been coincident outbreaks of influenza and of pneumonic plague. Notwithstanding this evidence, the epidemiological data relating to pneumonic plague over many years in many countries, and particularly the experience gained during the recent pandemic of influenza, render untenable the hypothesis that the influenza virus plays an important part.

Most authors, for example, Seyfarth (1925), Jorge (1926), Dujardin-Beaumetz (1926) and Wu (1926), can find no support for Norman White's views, which, indeed, can be traced in the literature of plague to comments made by the first Indian Plague Commission (1900–1), and to the bacteriological studies of the German and Austrian Plague Commissions. These early observations are open to criticism. Thus the increased mortality from diseases of the respiratory system, which was said to herald and accompany plague epidemics in Bombay, can be attributed, in part at least, to erroneous notification of the cause of death for the purpose of evading interference by the sanitary officials. Again the bacteriological records of post-mortem material, made by the early Commissions, suggest that, in some instances at least, agonal infections with streptococci and pneumococci, or post-mortem multiplication of these organisms had occurred. The work on fresh corpses exposed to low temperatures in the winter, which was carried out in Manchuria, is far more trustworthy.

The problem has two aspects, which need separate consideration: (1) the factors involved in the genesis of the first case of primary plague pneumonia from a patient with a secondary plague pneumonia, and (2) the mode of origin of the subsequent cases of primary pneumonic plague that make up the epidemic.

The first case of primary pneumonic plague in an epidemic is infected by droplets of sputum from a patient with a secondary plague pneumonia. Only a small proportion of the chances of implantation of the infection succeed, so that under average conditions only a small percentage of those exposed to risk are attacked by the primary form of the disease; reasons for this have already been adduced (see p. 210). Since a symbiotic virus as the exciting agent of the initial case of a pneumonic outbreak has not yet been demonstrated, it seems preferable, in the present state of our knowledge, to believe that the fortuitous origin of the first case is dependent upon a complex of factors which do not often concur. These factors are in all probability chiefly epidemiological, for example, overcrowding, bad ventilation and social habits and customs that favour transference of the infection; it is not unlikely that they include also a morbid state of the respiratory mucous membrane at the site of the implanted infection, or an unusual degree of susceptibility of the recipient's tissues to the infection.

As regards the reproduction of the primary pneumonic form, the pathological studies that have been carried out on material derived from the Chinese epidemics and from other sources make it certain that the plague bacillus is a lethal agent of such potency as to need no reinforcement from an associated virus. The enormous culture-mass in the lungs not only provides an abundant supply of plague bacilli in the sputum for doses that are destined to infect the entourage, but it also serves as a potential store of plague toxin, which is equivalent to many multiples of the minimal lethal dose for man, and which accounts for the rapid and invariably fatal issue. In the spread of the epidemic the factors that have already been mentioned as favouring the onset of the first primary pneumonic case still operate; and thus the magnitude of an outbreak, in whatever country it may occur, is determined by its particular set of co-operating factors and by the degree in which each is present (Petrie and Todd, 1923).

Clinical and post-mortem observations on an adequate scale in countries where outbreaks of primary pneumonic plague tend to occur, as well as in countries, like India, where there is little tendency to kindle pneumonic foci, are needed, in order to make clear the genesis of secondary plague pneumonia and its relation to primary pneumonic plague.

The Relation of Primary Pneumonic Plague in Man to Plague in Rodents.

Primary pneumonic plague in man can always be traced to plague in rodents. McCoy (1921) has stated that this form of plague in man is rarely associated with rat-plague, and that the definite tendency in the Californian ground-squirrel to pulmonary lesions is possibly significant. It is true that pneumonic plague is often derived from species of rodents other than the rat, such as the tarbagan, the spermophile and various rodents on the South African veld, but it is doubtful whether this relationship is more than an accidental one. In California, during the last 25 years, there has been only one outbreak of human pneumonic plague whose origin could be attributed to infected ground-squirrels; the only other outbreak occurred in 1924 in Los Angeles, and was of rat origin. Wu notes that in Madagascar, Portugal, and the Azores, where plague in rodents is confined to the rat, the incidence of human pulmonary plague is marked. The best example of this conjunction is afforded by the Qena-Girga District of Upper Egypt; this is a typical pneumonic plague region, and here *M. rattus* is the sole originator of the infection. The pulmonary lesions in the ground-squirrel are a characteristic feature of plague in this animal, and are in some degree due to the subacute nature of the infection; but it is difficult to understand why any relation should exist between the lesions in the lungs of ground-squirrels and the origin of human pneumonic plague.

Laboratory Infections.

A good many laboratory workers have died from primary pneumonic plague in consequence of the accidental inhalation of infective cultures or material, particularly in countries with a cold or temperate climate. Aerial infection in European laboratories is partly attributable to insufficient ventilation. Free ventilation helps to remove any infective material that may be present, and thereby lessens the risk to the worker.

THE PATHOLOGY OF PNEUMONIC PLAGUE.

Strong, Crowell and Teague (1912) concluded from their histological work on human and experimental material that epidemic pneumonic plague arises from inhalation of infective particles; and that the plague bacilli spread downwards from the site of implantation on the bronchial mucous membrane until they reach the infundibulum and air-cells, or pass through the walls of the bronchioles into the contiguous lung tissue, where they produce lesions of the lobular type, and later, by fusion, a lobar pneumonia. The bronchial glands at the bifurcation of the trachea show signs of an acute inflammation at an early stage of the disease. Wu's summary of the pathogenesis of primary pneumonic plague accords with these statements. He points out that fibrin in the alveolar contents is rare or absent, contrasting with the fibrin plugs that are found in croupous pneumonia ; a grey hepatization of the lung is not seen, because the patients die before this stage is reached.

Most workers consider that the septicæmia which so often accompanies primary pneumonic plague is secondary to the enormous multiplication of the bacilli in the lung tissues. In opposition to this view, Koulecha (1912) thinks that the septicæmia results from a primary lesion in the tonsil, and that the lesions in the lungs are of hæmatogenous origin. Strong and his colleagues agree that a primary infection of the tonsil may occur, but regard it as an uncommon event. The evidence is undoubtedly in favour of the correctness of the generally accepted opinion ; thus, histological examination shows that at an early stage of the disease the bacilli are present in the lymphatic tissues only, and not in the blood capillaries.

The symptoms in pneumonic plague are those of a toxæmia, and the fatal issue is due likewise to the action of the toxin of the plague bacillus.

Forms of Plague that may be met with in Pneumonic Plague Epidemics.

Cervical buboes from infection of the tonsils or pharynx, and buboes caused by accidental skin infection with sputum or with post-mortem material have been recorded. A nurse in a plague hospital in Bombay received a particle of sputum in the eye during a fit of coughing in a patient with pneumonic plague. Next day a conjunctivitis appeared, and this was followed by swelling of the parotid and cervical glands. Instances of bubonic plague in which the infection has been derived from patients with primary pneumonic plague are very rare.

In the Manchurian epidemic of 1910–11, Strong and Teague described as an exceptional occurrence a form of primary plague septicæmia, of which the site of origin was probably the tonsils or the mucous membrane of the mouth or throat. Heart symptoms were a conspicuous feature, and the course of the disease was rapid. Wu and his colleagues, towards the end of the Manchurian epidemic of 1920–1, observed a form of septicæmic plague with rapidly fatal issue, which at post-mortem showed only hyperæmic and œdematous changes in the lungs, and to which they gave the name 'pulmonary plague', in order to indicate that the infection had entered through the respiratory tract. It is to be expected that, just as in the bubonic type of epidemic, rapidly fatal septicæmic cases occur, depending presumably upon a high degree of susceptibility of the patient, so in pneumonic plague epidemics some of the patients will succumb to a rapidly progressive septicæmia before the pulmonary lesions have matured. Wu's suggestion that this type of case results from an enhanced virulence of the epidemic strain, owing to lung passage, and that the septicæmia may favour the spread of infection by means of human parasites, is rendered improbable by evidence from other sources.

According to Wu there is no satisfactory evidence that mild cases of primary pneumonic plague occur. Observers in the French colonies in Africa have described 'formes frustes' and atypical forms of primary pneumonic plague, but their statements have been criticized adversely. Thus, Heckenroth (Wu, 1926) pointed out the danger of mistaking plague-like bacilli in the sputum for *B. pestis*, and Dujardin-Beaumetz (1926) thought it important to distinguish clearly between primary and secondary pneumonic plague when reporting cases that appear to illustrate the occasional mildness and slight contagiosity of the primary form.

The Experimental Production of Plague Pneumonia.

Martini (1901) caused rats to inhale atomized plague cultures, and observed a primary plague pneumonia in 32 out of 36 experiments. The lesions were sometimes lobular, and sometimes lobar; pleurisy was frequently present. Strong and Teague (1912²) sprayed saline emulsions of plague bacilli for from 2 to 3 minutes into glass cages which contained experimental animals; 34 guinea-pigs and 55 monkeys were thus treated, and all died. Only about 23 per cent. of the guinea-pigs showed distinct evidence of pneumonia; on the other hand, infection of the cervical and laryngeal tissues was a conspicuous feature. Experiments on monkeys (*Cynomolgus philippensis* Geoff.) gave results which closely simulated the lesions found in man, because signs of infection in the cervical tissues were absent in nearly all; the tonsils were never primarily diseased, for they showed a moderate degree of congestion, and only a few plague bacilli were found in them; plague bacilli were always recoverable from the heart-blood. The lungs in every instance showed the changes that are characteristic of primary pneumonic plague; the lobular form was noted in the animals that died early, and the lobar form resulting from fusion of lobular areas in those which lived longer. Strong and Teague thought that the primary site of the infection was in some portion of the bronchi. The bronchial glands at the bifurcation of the trachea were always much more severely affected than the lymphatic glands elsewhere. According to these authors a primary plague septicæmia occurs as a rarity before any lesions are manifest in the lungs.

Two dogs died as the result of inhalation of plague suspensions; Strong and Teague conclude that dogs are only moderately susceptible, but that when exposed to a concentrated infection, they may contract primary pneumonic plague. Five donkeys proved to be insusceptible to this mode of infection.

Wu and Jettmar (1925–6) have described the histological findings in 20 tarbagans and 9 sisels (susliks) that were experimentally infected by the method of inhalation. The results confirmed these authors in their opinion that infection in plague pneumonia is not a faucial or even a tracheal one, but is an invasion of the deeper portions of the respiratory tract.

Pneumotropism of the plague bacillus.

Martini in the course of his inhalation experiments on rats raised the virulence of his strain by passage from lung to lung, and thought that the exalted strain, when inoculated subcutaneously or intraperitoneally, had a selective action upon pulmonary tissue. Polverini (1911) in similar experiments on monkeys in 1899 came to a like conclusion. The existence of such races has been assumed to explain the origin of pneumonic plague epidemics, but the observations on which the theory is founded need further study.

The Identity of Strains isolated from Cases of Pneumonic and Bubonic Plague.

The delegates to the Mukden Conference (1912) agreed that there was no fundamental difference between strains isolated from cases of pneumonic and from cases of bubonic plague.

The Virulence of Strains isolated from Cases of Primary Pneumonic Plague.

Strong and Teague (1912[3]) compared the virulence of 'pneumonic' and 'bubonic' strains by the method of cutaneous inoculation in guinea-pigs, and by the cutaneous, subcutaneous and inhalation methods in mice, rats and monkeys; there was little difference between the cultures in respect of virulence.

THE SPREAD OF PNEUMONIC PLAGUE.

The Mode of Infection in Pneumonic Plague: Droplet Infection.

In the pneumonic plague epidemic in the winter of 1905 at Oporto, Jorge (1926) exposed agar plates before the face of a patient, and obtained colonies of *B. pestis* up to a distance of 50 cm. In the Manchurian epidemic of 1910–11, similar experiments were performed. Thus, Strong and Teague (1912) exposed for about 2 minutes 35 plates at distances varying from a few inches to a few feet before patients who coughed, and plague colonies were found on 15; in some instances more than 100 colonies grew as the result of a single cough. The plates were seldom infected when no coughing took place: thus, there was only 1 positive plate out of 39 exposed. Strong thought that a vigorous cough might emit droplets to a distance of several yards. The time of exposure to droplet infection of persons in contact with a patient is an important factor in determining the chances of infection.

The Question of Carriers in the Spread of Pneumonic Plague.

The subject of carriers in pneumonic plague, whether the pneumonia is of the primary form or is secondary to bubonic plague needs critical revision, supported by fresh observations which will leave no doubt of the identity of the bacilli that are recovered. It would be well, too, if those who report such cases would send a subculture of the organism recovered to one of the various collections of type cultures. Many of the observations date from the early days of plague research, when less attention was paid to the exact identification of the plague bacillus. In a secondary plague pneumonia the bacilli may conceivably persist for some time after the patient is convalescent, but there is no record that such a carrier has ever passed on the infection to others so as to give rise in them to primary pneumonic plague. The evidence for healthy carriers in pneumonic plague epidemics is still more dubious, when it is considered that the disease is always fatal. The carrier-rate in respiratory diseases due to droplet infection varies inversely as the pathogenicity of the causal agent, and, therefore, the carrier in an epidemic of primary pneumonic plague is a negligible factor in its spread. Wu doubts whether there are racial differences in the immunity to pneumonic plague infection, but is convinced that some individuals possess sufficient natural resistance to protect them.

The Dispersal of the Infective Agent apart from the Pneumonic Plague Patient.

Most of the evidence under this heading is of a negative character, and the general statement may be made that plague bacilli, when expelled by the pneumonic plague patient in such a way as to contaminate objects in his immediate surroundings, do not survive as agents in the further spread of the infection. Thus, sick-rooms and houses are not infective apart from the plague patient, and it is unlikely that dust plays any part in spreading the infection. The pneumonic plague corpse is not a probable source of infection.

The Alleged Spread of the Infection to Rats and to Domestic Animals.

The chance of rats becoming infected from sputum or by feeding on human plague corpses is generally considered to be negligible, nor is there any reason for thinking that infection of domestic animals ever takes place in this way.

Social and Climatic Circumstances that predispose to the Epidemic Spread of Pneumonic Plague.

Overcrowding in badly ventilated dwellings, whether due to a low atmospheric temperature or to any other reason, is perhaps the most important factor. Close contact, and ignorance of precautionary measures against spray infection during the act of coughing by the patient, greatly increase the risk. The infection in many of the South African cases seems to have been conveyed by kissing; there is evidence that in some instances it has followed a meal of veld rodents, probably after very imperfect cooking; and it has been suggested that a plague pneumonia has supervened as a direct result of infection derived from the fleas of veld rodents. This suggestion seems to imply that the bacilli in flea fæces deposited on the skin may be transferred by the fingers to the mouth or nasal cavities: a speculation which hardly deserves credence. Many authors have laid stress upon the importance of a cold climate in the origin of pneumonic plague outbreaks. There is ample evidence of the association in the history of the disease, and yet it is certain that such outbreaks can also arise in hot, dry weather, as for example, in Upper Egypt during the hot season. In the Egyptian Plague Report mention is made of the tendency for a secondary pneumonia to supervene in persons who travel while they are suffering from bubonic plague, and it is suggested that the muscular efforts made by the patient increase the risk of detachment of infective thrombi which form in the blood-vessels in or near the bubo, with consequent metastatic inflammation of the lungs (Petrie and Todd, 1923). Seyfarth (1925) advances a similar view; he regards as important agents for the spread of the disease, ambulatory patients with sinuses or purulent wounds; these may persist for weeks or months after the bubo has broken down or has been incised, until finally, when the patient's resistance is weakened by any cause, they light up a septicæmia or a lung complication.

THE DIAGNOSIS OF PNEUMONIC PLAGUE.

During Life.

Examination of the sputum. Since the patient may have no cough it is necessary to encourage him to expectorate, so that a specimen may be obtained for bacteriological examination. A definite diagnosis is not possible during the first stage, which usually lasts for 24 hours (Wu, 1926). The characteristic, liquid, frothy, and pink sputum is obtainable in the second stage. A smear should be stained by Gram's method as well as by the ordinary stains. Involution forms are often seen, especially in the later stages. The sputum may be inoculated cutaneously into a guinea-pig.

Direct puncture of the lungs. This method is certainly worth using when it is important to secure a rapid diagnosis, as for instance, at the beginning of a suspected outbreak.

Examination of the blood. Blood-cultures may be negative early in the disease, but are almost invariably positive later on; the diagnosis can often be made by direct microscopical examination of the blood. Serological tests are not applicable; the brief duration of the illness does not allow time for the formation of antibodies.

After Death.

The German Commission advised that, when an autopsy could not be made, puncture of the lungs should be practised. Otten (1924) in Java thought that a positive lung puncture, together with a negative spleen culture, indicated a primary lung infection. Wu thinks that spleen punctures are as informative as lung punctures. Tsurumi (1924) states that the lower lobes of both lungs are to be preferred for lung puncture, because they are the parts of the lungs that are the most vulnerable to the infection; in his view, spleen puncture is not a successful method of diagnosis. The method of lung puncture was of great service in diagnosing primary pneumonic plague in Upper Egypt, and thus establishing the existence there of pneumonic plague outbreaks (Petrie and Todd, 1923). Smears of material taken by puncture from the apices and bases of the lungs, and from the heart-blood, generally gave incontestable evidence of pneumonic plague; the preparations often resembled those made from a pure culture. In outbreaks of pneumonic plague the early cases are often overlooked, even in countries that possess skilled public health and medical services. This simple and safe procedure cannot fail to be useful wherever there is reason to suspect that pneumonic plague may have been the cause of death.

IMMUNIZATION AGAINST PNEUMONIC PLAGUE.

Experimental Vaccination against Pneumonic Plague.

A satisfactory degree of protection in actively immunized animals has not yet been attained. Strong and Teague (1912) worked with monkeys that had been previously immunized with a living avirulent culture; of 54 animals, only 22 per cent. survived the inhalation of a virulent culture as compared with 7 per cent. of 55 control animals. Guinea-pigs similarly treated showed much more favourable results—71 per cent. and 3 per cent. are the corresponding figures—but the experiments are not admissible as proof of protection against pneumonic plague, because, as Strong and Teague discovered, guinea-pigs seldom contract experimental pneumonic plague. These authors conclude that prophylactic inoculation cannot be relied upon as a means of protection against pulmonary infection in man. Wu, however, argues with justice, that experimental conditions are necessarily artificial, and not altogether comparable with natural infection by sputum droplets. The dosage and the degree of atomization of the infective particles, whether of cultures or of sputum, no doubt have an influence upon the chances of infection.

Experimental Serum Therapy in Pneumonic Plague.

Martini (1902) found that anti-plague serum completely protected rats when given in a dose of 1/60th of the body weight 1 hour before the experimental inhalation of plague bacilli, or during the incubation period, that is, within 20 hours after inhalation; the protective effect lasted only a few days. The serum had scarcely any curative effect in rats which were suffering from pneumonic plague, although it perhaps prolonged life. The serum did not protect mice, guinea-pigs, rabbits, and cats.

Vaccination in Man against Pneumonic Plague.

Prophylactic plague vaccines were used on a considerable scale in the Manchurian epidemic of 1910–11, but the delegates to the International Conference at Mukden (1912), who weighed the evidence, were unable to come to any definite conclusion upon their efficacy. There were occasional instances of infection in spite of prophylactic inoculation, and although it is likely that vaccination is not wholly trustworthy, it seems best to use the method, especially for persons who are exposed to prolonged contact with the infection; they should, however, be warned of its limitations.

Serum Prophylaxis against Pneumonic Plague in Man.

Wu has reviewed the rather scanty data on this subject, and concludes that anti-plague serum, when given prophylactically, often prolongs the incubation period, and gives the subsequent treatment with serum a better chance of success. Dr. Sachs died at Berlin of pneumonic plague as the result of a laboratory infection; 25 c.cm. of serum were given to his attendant as a prophylactic dose; he contracted the disease, but recovered after treatment with 155 c.cm. of the serum. Wu advises that the serum should be given as a precaution whenever a risk of lung infection has been incurred, as in post-mortem work, and after laboratory accidents; a dose of vaccine may be given in addition to the serum.

Serum Therapy in Pneumonic Plague in Man.

The results that have been reported are disappointing, but there are indications that some benefit has followed the use of anti-plague serum in pneumonic plague. There is evidence that by its use life is at least prolonged, and that some of the symptoms are ameliorated within a few hours of its administration, a result which suggests that the serum has an antitoxic action. The early administration of large doses of serum by the intravenous route is essential if any useful result is to be obtained. Research on the preparation and standardization of anti-plague serum is necessary before its clinical use can be put upon a sound basis. In particular it is desirable to agree upon a test for the measurement of antitoxin content in terms of a standard unit, and to ascertain whether any other antibodies are essential to the therapeutic efficacy of the serum.

THE PREVENTION OF PNEUMONIC PLAGUE.

Disinfection of the Sputum.

The plague bacillus is not very resistant to the action of disinfectants, but the coagulant action of some of these substances on the albuminous matter in the sputum may assist its survival by protecting it from direct contact with the disinfectant. Wu believes that plague sputum is rendered innocuous by the action of direct sunlight.

The Segregation of Contacts.

Persons who have been in contact with pneumonic plague patients should be separated into at least two groups according to the degree of exposure to the infection, and since pneumonic plague outbreaks may arise among contacts, it is better to arrange, when possible, for their segregation into still smaller groups. Systematic medical observations, including temperature records, should be made, so that those who show even the slightest signs of illness may be further isolated. The quarantine period should extend for seven days after the last contact with the plague patient.

Personal Prophylaxis by means of a Mask.

Attendants on the sick must wear a mask over the nose and mouth, and the eyes should be protected by goggles. In the first Manchurian epidemic a simple mask was worn, which consisted of a pad of cotton-wool enclosed in a broad gauze bandage, sufficiently long to be tied round the head. Some workers prefer a hood or helmet of flannel, or similar material, which is fitted with a celluloid or a mica window. Barber and Teague (1912), as the result of spraying experiments with *B. prodigiosus*, doubted the effectiveness of the wool and gauze mask, and thought that it can afford only a partial protection. However, experience in Manchuria has confirmed its utility, and moreover, as stated by Wu, spraying experiments are perhaps not altogether relevant to the actual conditions met with in pneumonic plague epidemics. A disadvantage of most forms of mask is that the filtering material becomes wetted with the moisture of the breath, and so loses its efficiency ; this can be overcome by arranging for an exit valve for the expired air. By incorporating useful ideas that have been gained from experience with the gas mask, it should be possible to devise a comfortable and efficient mask for protecting those who are exposed to concentrated infection in pneumonic plague hospitals or elsewhere.

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CHAPTER IX. THE ORGANISMS ASSOCIATED WITH GAS GANGRENE.

BY MURIEL ROBERTSON
(LISTER INSTITUTE, LONDON).

WITH A SECTION BY R. A. O'BRIEN.

History of the Bacteriology of Gas Gangrene.

PASTEUR, in 1863, laid the foundation of all the work on the anaerobes that was to follow by his discovery of *B. butyricus*, a non-pathogenic, saccharolytic, spore-bearing anaerobe. (Pasteur, 1861[1 & 2]; 1863.)

It is necessary to give some account of the sequence of events in the bacteriological study of the anaerobes in order to obtain a key to the almost fantastic chaos of the literature and of the nomenclature. It must be noted that the subject contains within itself the kind of difficulty that leads to confusion. It is more difficult to obtain pure cultures and to maintain them in the pure state than in other branches of bacteriology, and, as will be seen in the sequel, microbic associations of a very stable nature can arise which simulate cultural entities, but which are none the less mixed cultures. The cryptic proliferation of one anaerobe actually within the characters of another, and the tendency, indeed the preference for growth in mixed colonies rather than in separate ones, constitute a quite special series of difficulties which are even now insufficiently appreciated.

B. chauvœi was discovered by Bollinger (1875) and by Feser in 1876, and was cultivated in 1879 by Arloing, Cornevin and Thomas. (See also 1887.) In spite of the fact that it was often studied in mixed cultures and also in spite of much confusion with other organisms, it is universally recognized now as a bacteriological entity and its characters are agreed upon; it has, moreover, preserved its original name although it has been the centre of many controversies. *B. chauvœi* was well described by the early authors (Kitt, 1887; Roux, 1887); Roux and Chamberland (1887) got evidence of a soluble toxin, and Kitasato cultivated it on solid media in 1890 (Kitasato, 1889 and 1890).

In 1877 Pasteur isolated in liquid media the organism which he named 'vibrion septique' (Pasteur and Joubert, 1877[1]). There is some slight confusion in his account of the condition produced in experimental animals, and there is evidence that he may have been dealing with mixed cultures at times, but what he finally added to the bacteriology of his day were pure cultures with clearly defined characters.

Koch (1881) also discovered a pathogenic anaerobe which produced a fatal œdema, but his description is too full of confusion to be of much value at the present time.

Novy, in 1894, described what was then a new type of malignant œdema bacillus; he got pure cultures and it emerges as a well-defined organism. It was rediscovered during the war and was the subject of detailed study by Weinberg and Séguin under the name of *B. œdematiens*. In 1892 *B. welchii* was described by Welch and Nuttall as *B. aerogenes capsulatus*, and in the next year (1893) Fraenkel dealt with it as the gas phlegmon bacillus and it was named once more in 1898 as *B. perfringens* of Veillon and Zuber.

Tissier and Martelly (1902), in a study of the putrefaction of butcher's meat, added to the understanding of the variety of forms to be found in the general group of the anaerobes. Metchnikoff in studying the intestinal flora (1908) drew attention particularly to the putrefactive group and the name *B. sporogenes* dates from this work.

Von Hibler's book appeared in 1908. In this book and in the articles of the following years a type known as *B. œdematis maligni* Koch was described, which had as its leading character that it was a putrefactive organism which digested milk and blackened meat medium and gave off a putrefactive odour, it was pathogenic but the pathogenicity might lapse with cultivation. This unfortunate description has done more to confuse the work upon the pathogenic anaerobes during the last fifteen years than is credible, unless its trail is actually followed. This type was in effect a mixed culture of a non-pathogenic, proteolytic bacillus of the *B. sporogenes* group and some one or other of the pathogenic malignant œdema organisms. Most unfortunately von Hibler fathered this proteolytic type of culture upon Koch. He also, however, recognized Ghon and Sachs' malignant œdema bacillus quite correctly.

After von Hibler's book there were in the field *B. welchii*, *B. chauvœi*, *B. novyi* and the three names, 'vibrion septique', 'malignant œdema' of Ghon and Sachs (1903[1 & 2], 1904[1 & 2]) and 'malignant œdema' of Koch or Koch-Hibler. In the German literature for many years after this date the term 'bacillus of Ghon and Sachs' is used for all the non-putrefying, that is, for all the pure, cultures of an organism which agrees in its characters with vibrion septique; *B. œdematis maligni*, Koch or Koch-Hibler, is used for a proteolytic malignant œdema, which in the opinion of the writer has never been shown to be a pure culture. Quite recently Zeissler has also come to this conclusion and no longer admits a putrefying *B. œdematis maligni*. The term *B. œdematis maligni*, Koch, was also used for a time (Robertson, 1916), in error arising out of the foregoing, for non-pathogenic cultures which were in effect *B. sporogenes*.

For reasons given below *B. œdematis maligni* Ghon and Sachs will be used for the non-proteolytic highly pathogenic organism first named vibrion septique. Liborius (1886) seems to have been the first actually to use the latin form *B. œdematis maligni;* he noted, moreover, that pure cultures have no putrefactive odour.

None of these four important pathogenic organisms so far mentioned escaped the vicissitudes of the mixed culture, and the confusion and the controversies that these discrepancies occasioned fill the journals.

The value of these disputes at the present time lies in the illustration they afford of the importance of pure cultures, and also in the recognition that the early serological deductions suffered necessarily from the confusion in the bacteriology of the group.

The history of the work on the anaerobes during and since the war is summarized under the appropriate headings in the various sections of the chapter, and need not, therefore, receive special reference.

General Outline of the Characters of the Bacteria.

The organisms involved in gas gangrene belong to a well-defined group. They are characterized by growth under conditions of greater or less anaerobiosis, they are Gram-positive rods and form resistant spores. The morphology includes pleomorphic forms of great variety. The growth may cause the active fermentation of sugar, the clotting of milk, the digestion of protein and to some extent the splitting of fats; in addition, soluble toxins of great interest are formed by some members of the group. In general, it may be said that all the gas gangrene anaerobes are primarily saprophytic bacteria living in the intestine of animals and proliferating also in decaying animal and vegetable matter. The great resistance of the spores, which are formed in large numbers, leads to their survival for long periods in earth, and to their wide dissemination in dust, so that they are almost universally present as contaminating organisms.

CLASSIFICATION.

The anaerobes are classified according to their morphology and according to certain of the cultural characters. Table I gives an arrangement of the group based upon the nature and position of the spores and in relation to the proteolytic and saccharolytic growth reactions. This classification is that drawn up in the Medical Research Committee report of 1919, and seems still to arrange the data in a satisfactory manner.

In work upon the anaerobes it must be recognized that species have a group nature and that certain types of criteria are taken as delimiting species, while other criteria can subdivide the species into races. In the anaerobes agglutination is one of the characters that may be considered to be ultraspecific, the reasons for this are discussed later (p. 284). The formation of a toxin with uniform antigenic properties is used as a specific character. This is a perfectly arbitrary choice, but it is convenient and has certain reasons to justify it which will be set out in a later section (p. 262). The uniform toxin thus binds together the races of *B. œdematis maligni* (vibrion septique) which can be subdivided by agglutination tests, and the races of *B. welchii*, which can be classified upon the fermentation of inulin and glycerin (Simonds, 1915[2]). In the nomenclature adopted

TABLE I.

	Both proteolytic and saccharolytic properties.		Slight proteolytic but no saccharolytic properties.	Saccharolytic but no proteolytic properties.	Neither saccharolytic nor proteolytic properties.
Morphology.	Proteolytic properties predominating. Coagulated serum and gelatin are liquefied.	Saccharolytic properties predominating. Serum not liquefied; gelatin liquefied.	Serum not liquefied. Gelatin liquefied.	Neither serum nor gelatin liquefied.	Neither serum nor gelatin liquefied.
Central or sub-terminal spore.	*B. sporogenes.* *B. parasporogenes.* *B. centrosporogenes.* *B. bifermentans.* *B. aerofœtidus.* *B. histolyticus.*	*B. welchii.* *B. œdematis maligni* (vibrion septique). *B. chauvœi.* *B. novyi.*		*B. fallax.* *B. multifermentans tenalbus.*	
Oval terminal spore.				*B. tertius.*	*B. cochlearius.*
Spherical terminal spore.			*B. tetani.*	*B. tetanomorphus.* *B. sphenoides.*	

here there is admittedly a tendency to avoid undue splitting. Single or very rare strains are not given very much attention, although the more important ones are mentioned when sufficiently described.

The names employed need some justification. *B. welchii* is used for *B. aerogenes capsulatus* because such use is sanctioned by custom and because that name has always denoted the same bacterial entity. Synonyms are Fraenkel's bacillus, of the German literature, and *B. perfringens*, of the French. *B. novyi* is used because Novy described the bacillus very clearly and it has had a relatively undisturbed history. A synonym is *B. œdematiens* of Weinberg.

The title *B. œdematis maligni*, Pasteur and Joubert and Ghon and Sachs, is used with some reluctance. The organism is the vibrion septique and this name has priority, and would certainly be preferred by the writer because of its much clearer record if it were a name in the botanical sense at all. Ghon and Sachs gave a perfectly sound description of their bacillus under the name of *B. œdematis maligni*, which is true and recognizable to-day. They correctly admitted that it was identical with Pasteur's vibrion septique and they had the right to adopt the botanical name first used by Liborius in 1886 and affix it to the type studied by them.

It should be noted that Koch, in calling his organism 'Bacillus des malignen Œdem', was using a trivial name just as Pasteur was. Ghon and Sachs had certainly rediscovered the organism that Pasteur had found and probably the organism of Koch, and though their name has at various times been incorrectly applied, nevertheless it is the correct procedure to use it for this organism. Another reason in its favour is that it is current at least in English, American and German literature.

Heller (1921[1 & 2]; 1922) gives a classification of the anaerobes that dispenses entirely with all the traditional names. This seems inadvisable, as it renders almost incomprehensible a lot of excellent and fundamental work and cuts the literature in two from the date of its adoption. The classification in itself seems to be unduly complicated.

Zeissler's emended classification, as communicated to the writer in 1926, agrees in matters of fact with that given here, except that he is doubtful about the position and identity of *B. fallax*, *B. bifermentans*, and *B. multifermentans tenalbus*. The names he uses are given as synonyms in the particular descriptions of the individual bacteria, as they represent the titles current in German literature.

BACTERIAL NATURE OF GAS GANGRENE.

Gas gangrene is a generalized disease incidental to infection with one or more of the pathogenic anaerobes. It must be understood that it is not an inevitable sequel to such infection, it is an incident which occurs when a given set of conditions arise. The proliferation of very pathogenic forms within a wound may lead only to a heavy local infection with gas and a local necrosis, or it may produce a fulminating gangrene of such violence that death ensues within a few hours.

The particular anaerobe which causes this generalized condition may be the only one which is invading the sound tissue, but in wound gangrene it is certainly never the only anaerobic organism present in the wound. Gas gangrene may be due to two or even three different pathogenic anaerobes; in addition, non-pathogenic species may be carried into the blood stream and may be recovered from this situation during life. The presence of anaerobes in the blood stream is a more common accident than is generally appreciated (Stokes, 1915 and 1916), and may even occur in cases of heavy local infection where there is little or no general toxæmia.

The initial infection in wound gangrene is nearly always set up by a group composed of several quite distinct anaerobic organisms. The truly non-pathogenic forms, while multiplying in the wound, are unable by themselves to penetrate the sound tissue, but they may set up conditions which are favourable for the proliferation of the pathogenic types, and may influence the character of the gangrene should this supervene. The pathogenic anaerobes of this group are at once penetrating organisms that proliferate in the tissues and may circulate in the blood stream, and, in addition, they produce soluble toxins that are extremely poisonous; it is the toxæmia that is apparently the cause of death in gas gangrene. The action of the toxins is analysed in a later section (p. 261).

It is important to realize that the whole cycle of manifestations found in wound gangrene is only one of the possible complexes of symptoms that may be set up by these organisms. Wound gangrene is the sequel to a wound, and the organisms are lodged in the tissues and set out from that site as the starting point. They can, however, produce their effect in quite other ways; it is an erroneous conception to think of anaerobic infections as purely muscle diseases; the changes produced by these bacteria may take place in any organ. Moreover, it must be remembered that all the anaerobes can and do proliferate under suitable conditions in the hollow viscera. The toxic effects of the pathogenic forms can be exercised at any site where the environment is favourable and where toxin can be absorbed.

Morphology and Cultural Characters.

The list of anaerobes considered here is as follows; they are placed in the order of their importance in gas gangrene and in the diseases of animals:

B. welchii.
B. œdematis maligni (vibrion septique).
B. chauvœi.
B. novyi (*B. œdematiens*).
B. fallax.
B. histolyticus.

Certain organisms considered to be pathogenic, but unnamed or only described from single strains, are briefly mentioned:

B. sporogenes.
B. parasporogenes.
B. centrosporogenes.
B. bifermentans.
B. aerofœtidus.
B. tertius.
B. multifermentans tenalbus.

MORPHOLOGY.

The anaerobes of the gas gangrene group are all rods, at least during the greater part of their active life. They are of relatively large size, the smallest bacillary elements being about 2μ in length and the longest single rods, as distinct from filamentous forms, reaching to about 8μ. They all form spores at some time. Certain species show a very high degree of pleomorphism under particular conditions of growth. The best example of this is afforded by *B. œdematis maligni.* Long filaments reaching to 40 or 50μ in length may be formed, deeply staining bulb-like and club-shaped forms occur singly or even in chains, and the curious 'citrons' and 'navicular' types are also found. These last are pale citron- or boat-shaped bodies with deeper staining points at one or both extremities. Some species, notably *B. welchii,* show a tendency to a granular type of staining; this represents a condition that can be recognized in living bacilli. All these anaerobes are Gram-positive in quite young cultures; there is, however, a tendency to be Gram-negative or Gram-labile in older cultures. This variability in the staining reaction is more marked in some species than in others, but is a character of the whole group. Flagella are present in many of the forms; capsules are found in *B. welchii,* but have not been demonstrated in any other species.

CULTURAL CHARACTERS.

It seems advisable at this point to make clear the attitude taken towards the fantastic degree of variation claimed for many of these anaerobes by certain authorities (Grasberger and Schattenfroh, 1901; Conradi and Bieling, 1916; Kolle, Ritz and Schlossberger, 1918; Ghon, 1917, &c.).

The phenomena noted by the workers cited above, and by their followers, appear to be due to the impurities of their cultures.

Media. (See Vol. IX.)

Anaerobiosis. All the bacteria in the group dealt with here require some degree of anaerobiosis for their growth and development. Some are extremely strict anaerobes, others are less exacting, while the only species that is capable of some actual degree of surface growth in the presence of air is *B. histolyticus* (Hall, 1923[1]).

(For further information on anaerobiosis and respiration, see Vol. I.)

Temperature. Most anaerobic bacteria are able to grow through a relatively wide range of temperature; 37° C. is in most cases the optimum temperature, but growth will take place up to and even above 45°, and may also go on slowly at relatively low temperatures such as 18°. There is a group of thermophilic anaerobes which develop at 55°; some of these will also carry on all their reactions at 37°; *Cl. thermoputrificus* is an example (Damon and Feirer, 1925).

Characters of the colonies. The characters of the colonies formed by the anaerobic bacteria in surface growth, and in deep agar shakes, have been used as one of the means of distinguishing them from one another. In the hands of an experienced worker the colony is undoubtedly a very useful character, but there is a rather wide range of normal variation according to the exact conditions of growth, so that too much emphasis should not be laid upon it as a final means of distinction. Zeissler (1922 and 1923), while having done an excellent service to the study of the anaerobes, tends to exaggerate the importance of the colony as a single decisive character.

In surface growth there are three main types: (i) The round or slightly crenated colony, which may be opaque as in *B. welchii*, or translucent as in *B. tertius*; this type, when pure, never penetrates downwards into the agar. (ii) The round colony with crenated edges which may grow out into fern-like processes on a moist plate; *B. œdematis maligni* is a typical example of this. (iii) The woolly encrusted colony which sends roots down into the agar; this is exemplified by *B. sporogenes*. The first type of colony is lenticular and smooth in deep agar, the second is delicate and branching, and the third is woolly and tangled. It should be remembered that there is a tendency in most anaerobes to form delicate spreading growths all over the surface of the plate. The anaerobes have also an inclination to grow in mixed colonies, where more than one type is present in the inoculum. This cannot be too much emphasized.

Behaviour of the anaerobes in various media. The cultural characters of the various anaerobes are of great importance in distinguishing the species from one another. Many of the reactions are accompanied by macroscopic changes in the media which can be read with ease. It should be mentioned that changes in the media go on for much longer in the case of the anaerobes than with other bacteria; their full extent cannot be estimated under 10 to 15 days' incubation. But any change that only begins to appear very late in the period of incubation (after 15 to 20 days) should in the first place be considered as probable evidence of a mixed culture, and the results carefully criticized along those lines before any conclusions are drawn. While parallel cultures should be incubated for the long period mentioned the readings can be taken after 24 to 48 hours' growth. For certain types of work very young cultures of only a few hours' growth are very advantageous.

Fermentation of sugars. (See pp. 234 et seq.)

Selective substances. (See pp. 234 et seq.)

Latency and microbic associations. Latency, which is a well-marked feature of the behaviour of the anaerobes, is probably related to the production of the appropriate oxygen tension in the medium (see Quastel and Stephenson, 1926).

Microbic associations occur between two or more anaerobes, and cultures are formed at times in which the balance is so perfect that none of the ordinary methods, such as plating and subculture, serve to upset the equilibrium. These mixed cultures simulate bacterial entities and may mislead even experienced workers. A frequent and very embarrassing form of microbic association, usually between an alkali-producing proteolytic form and a saccharolytic type such as *B. sporogenes* and *B. welchii* occurs, in which the proteolytic form is held in subjection. It may be passed over, as all the macroscopic reactions will be those of *B. welchii*, and microscopic study will reveal no significant mixture at this stage. That the colonies are mixed will, however, be shown after a time by a slight pitting of coagulated serum, and by a slight sign of rootlets or dark threads in the heart of the older and larger colonies after 5 to 10 days' incubation. Serum agar on account of its transparency is an excellent medium to reveal the rootlets or the slight darkening. Prolonged incubation on coagulated serum and subculture from serum to serum will presently produce liquefaction, but even at this stage no attempt at all at growth in independent colonies will be made. It is a work of almost incredible difficulty to grow out the subjected culture, though the continued growth upon protein media will so alter the character of the association that the fact of the admixture becomes plain. The opposite effort is somewhat easier, namely, the purification of the ascendant type, and this is fortunately usually the direction of the bacteriologist's endeavour.

Primary isolation. (See Vol. IX.)

Resting cultures. One of the important characters of anaerobic bacteria is the long survival of cultures after the period of active proliferation is over. Cultures in suitable media which have gone over into this resting state may be sealed by fusing the glass of the tube in the blow-pipe and preserved for years. On opening the tube and seeding into culture media, preferably into chopped meat or liver meat, or chopped meat with glucose or egg, vigorous growth will be produced and the strain can be carried on in a state of activity once more.

The writer has carried on from a sealed meat culture of *B. chauvœi* of eight years' standing, and from a *B. welchii* culture in alkaline egg-fluid after ten years, also from a culture of *B. histolyticus* in casein broth with a piece of coagulated egg-white after eight years; the particular times are the accidents of an individual experience and do not denote the limits of survival.

It is necessary to appreciate the particular needs of the different species in the matter of obtaining conditions of long survival, these are noted in the sequel. It is an obvious advantage to seal off a large number of well-tested cultures for future reference. The behaviour of a strain upon being subcultured after a prolonged period of quiescence is sometimes an interesting comment upon supposedly pure cultures.

Description of the Individual Bacterial Species.

B. WELCHII.

B. welchii Migula, 1900. (Synonyms: Bacillus of Achalme, 1891; *B. aerogenes capsulatus* Welch and Nuttall, 1892; *B. phlegmonis emphysematosæ* Eug. Fraenkel, 1893; *B. perfringens* Veillon and Zuber, 1898; *B. enteritidis sporogenes* Klein, 1895.)

Motility. *B. welchii* is non-motile under all conditions.

Morphology. The rods are 4 to 8μ in length, but short, almost coccoid forms may be present where division is occurring very rapidly, and filaments of several elements may be found under some conditions. The bacilli are Gram-positive in quite young cultures, they are usually straight with somewhat square ends. A granular indented appearance may be seen and many Gram-negative elements are present in slightly older cultures. Curved rods are found in certain strains, especially upon serum-containing media. *B. welchii* is a capsulated organism; the capsule cannot be demonstrated equally well in all culture media, but it is always present and can be made evident by transferring the rods into serum or into a weak solution of sodium hydrate (Robertson, 1916). There are no flagella. Pleomorphic types are produced upon coagulated serum, and to some extent in most serum-containing media. The range of forms is very wide: long filaments, club shaped, tadpole forms and granular types are all produced.

Spores. Spores are not formed upon all media; they may occur in very small numbers in the meat medium, but they are so few in number that the evidence for their existence is obtained from the resistance of the culture to heating, not from the microscopic examination. They occur upon coagulated serum and Dorset's egg medium, and in alkaline egg-fluid. There is some degree of variability in the readiness to form spores even in suitable media in different strains, but in the experience of the writer spores are formed in pure cultures with very rare exceptions, if good growth has been obtained upon coagulated serum or in alkaline egg-fluid. The spores are relatively large, they are oval with slightly flattened ends and are central or subterminal in position. Once the spore is developed the rod seems to disintegrate rapidly and the spore-bearing rod is not a very frequent appearance. The spore will resist heating to 80° C. for more than an hour; they will resist 97 to 100° for 1 to 3 minutes, but the nature and reaction of the medium surrounding them influences the survival time.

Anaerobiosis. *B. welchii* is one of the less exacting types in the matter of anaerobiosis and will grow under less perfect conditions than the absolutely strict anaerobes, such as, for example, *B. chauvœi.*

Colony. Surface colonies on nutrient agar, serum agar, or glucose agar, are round; at first translucent and later granular and more opaque, they may grow in 48 hours to 1 or even 2 mm. in diameter; if the plate is not crowded, the edges may be crenated. The colonies never send rootlets down into the medium and little dark clumps in the substance of the colony is always a sign of contamination. Deep colonies are lenticular.

Cultural reactions.

Meat medium. Very rapid growth, gas and pinkish colour. The reaction becomes acid, there is no blackening and there are no macroscopic signs of digestion. The odour is acid but not putrefactive.

Milk medium. Rapid formation of acid and clot, the clot is torn by the gas evolved, producing the well-known stormy fermentation. A few strains otherwise typical were found to exert a less vigorous action upon milk. These strains were very pathogenic, their serological reactions were those of *B. welchii.*

Coagulated serum. No macroscopic change, no liquefaction, spores are formed, pleomorphic types and filaments occur especially in old cultures.

Alkaline egg-fluid. The medium becomes evenly opaque but is not precipitated in large floculi nor is a clot produced. Spores are formed upon this medium.

Gelatin. The medium is liquefied.

Broth. The medium becomes turbid and gas is formed, after 24 to 48 hours the organisms sink to the bottom of the tube, the reaction becomes acid.

Substances fermented. Glycerin (variable), glucose, lævulose, galactose, maltose, saccharose, lactose, inulin (variable).

Substances not fermented. Mannitol, dulcitol, salicin.

B. welchii is hæmolytic.

Glucose broth is valuable for the great rapidity of growth obtained, but should only be used for this purpose and for very rapid subculture, as the degree of acid evolved kills the culture. Milk should be employed somewhat in the same way, but in general is better kept as a collateral test than used for the main line of propagation. Cultures should be maintained in the growing state upon one of the chopped-meat media or in brain brew. They are best preserved in the resting state in alkaline egg-fluid or coagulated serum, coagulated egg or casein broth, with a large piece of coagulated egg-white. Care should be taken that good growth has actually taken place before sealing the tube. The media of choice will, of course, be the alkaline egg-fluid and the casein broth with the piece of egg-white, as they are autoclaved in the final stage of preparation. On undertaking any work with a resting culture (i.e. in the form of spores) the first subculture should be made into meat with or without the addition of glucose broth. A second subculture should then be made, using the plain meat medium, and any subsequent work carried on from that. For any test such as growth in sugar, test of the pathogenicity or seeding for the production of toxin, a routine method should be adopted such as a double subculture at 24 to 48 hours' interval into meat to ensure an actively growing culture. For the preservation of virulence during a long period, the cultures should be made on serum or in alkaline egg-fluid and sealed. *B. welchii* strains show variations in pathogenicity; there is a very marked difference in this quality in different strains, but there is also a wide apparent variation due to conditions of culture.

B. welchii is divided into four groups (Simonds, 1915[2]) on the fermentation of inulin and glycerin. One toxin is produced by all the four types and they are treated as cultural groups within one species. During the war the writer became assured of the existence of these groups first described by Simonds, but Kahn (1924), working with a very large number of strains, while he also confirms Simonds' observations, finds, in addition, that there are strains that seem to bridge the types: intermediate gradations where some fermentation of one or both substances removes the sharp demarcation of the groups.

The soluble toxin which is produced by *B. welchii* is discussed on p. 262.

Agglutinins have not been demonstrated for *B. welchii* by the injection of washed bacilli into the blood stream of rabbits.

B. EGENS.

This is a type of *B. welchii* with poor fermentative capacity. A strain was isolated by Stoddart, who considered it to be a separate species. It is pathogenic for laboratory animals, and the writer finds that the serum derived from normal *B. welchii* cultures protects animals against infection. This was confirmed by Dalling in 1927 (unpublished communication).

B. ŒDEMATIS MALIGNI.

B. œdematis maligni Liborius, 1886; Gohn and Sachs, 1903. (Synonyms, vibrion septique, Pasteur and Joubert; *Cl. œdematis maligni* Bergey, 1923; Pararauschbrand Bacillus, Zeissler, 1923.)

B. œdematis maligni, as the name is used here, is identical with vibrion septique; it is a species with several subspecific divisions (see *infra*). It is the causal agent of serious or fatal gas gangrene and of septicæmia under certain conditions in man; it is also found as the infecting organism in a number of diseases in animals, such as braxy, symptomatic anthrax in hogs, and a disease which bears a clinical resemblance to blackleg. *B. œdematis maligni* is distinct from *B. chauvœi* (the causal agent of true blackleg) serologically, culturally and morphologically; they resemble each other, however, and the confusion of the two organisms is explicable. In the German literature, Kitt's Rauschbrand bacillus and the bacillus of 'Geburtsrauschbrand' appear to belong to the species *B. œdematis maligni* in the sense used here.

The putrefying actively proteolytic and pathogenic strains described by German workers do not, as described, belong to this species, and the writer has never come across a pure strain of this type. This was also the experience of Weinberg and Séguin and other workers in France during the war. The post-war research in America is in general agreement with this body of work. The writer is of the opinion that the pathogenic element in these putrefying cultures may in some instances be *B. œdematis maligni*, in which case the putrefactive characters are due to a proteolytic contaminating anaerobe. Zeissler in his latest work has also come to this conclusion.

Motility. The organism is motile in quite young cultures and in the exudate from wounds and infected animals.

Morphology. B. œdematis maligni is a Gram-positive rod of varying length and more slender than *B. welchii.* Spores are readily formed, and are an obvious character of active cultures of any age, though they may be absent sometimes in very early or poorly growing cultures at the first examination. The spores are oval and are usually subterminal, but central spores are also frequently seen, especially in certain strains ; the rods may grow in chains.

There is a relatively wide range of morphology even in the meat medium, but upon serum or in fluids to which fresh tissue has been added this tendency reaches its maximum. The dark-staining bulb-like forms, which may grow in chains, are often seen in young meat cultures, as also the club forms. In addition to these the 'citron' or 'navicular' types, in which the body is pale with a deep staining part at one or both extremities, and long filaments are found upon coagulated serum and tissue-containing media. The navicular forms may be met with in material taken directly from infected tissues and in blister fluid, &c. In material from artificially infected animals the pleomorphic types can all be seen, but spores are not as a rule found until late in the infection, in many cases only after death. Certain strains may have slight peculiarities, such as a greater or less tendency to grow in chains or to form central spores, or there may be a more frequent production of the dark bulb types in young meat cultures. These faint variations were noted among strains even in the same serological group and of uniform behaviour in other ways.

The spores will survive exposure to 80° C. for more than an hour and 97 to 100° for 5 to 15 minutes, according to the nature of the medium.

Anaerobiosis. B. œdematis maligni is a strict anaerobe and requires good conditions in this respect if satisfactory surface growths are to be obtained.

Colony. Surface colonies can be obtained upon plates, upon the surface of serum agar or blood agar, with or without the addition of glucose. Serum agar plates to which 1 per cent. of salicin has been added give a quicker and more prolific growth. The colonies are transparent and faintly opalescent, with smooth or indented or crenated edges according to the moisture of the surface, the length of growth and the vigour of the different growth periods. The older colonies may be opaque, especially on salicin agar. There is always a marked tendency to grow in a spreading surface film. Colonies in deep agar shakes are delicate and branching; the centre of the colony is not as a rule compact.

Cultural reactions.

Meat medium. Gas is formed, the colour is pink to bright red with no blackening. The odour is somewhat rancid but not putrefying. There is often a tendency for the supernatant broth to become quite clear after 4 to 6 days.

Milk medium. Acid is produced and some degree of clot; gas may be formed. The changes in the medium are slow, and the clot does not as a rule appear before 3 to 6 days. Occasionally there is poor growth in milk.

Coagulated serum. No liquefaction takes place; the morphology of the organism is very variable.

Alkaline egg-fluid. The medium is rendered more opaque; there is no clot and no digestion.

Gelatin. This medium is liquefied.

Broth. The medium becomes turbid, and after a period of growth the organisms sink down to the bottom of the tube, leaving the supernatant fluid perfectly clear.

Substances fermented. Glucose, lævulose, galactose, maltose, lactose and salicin.

Substances not fermented. Glycerin, saccharose, inulin, mannitol and dulcitol.

Some workers (Kahn, 1925) consider that certain strains ferment saccharose, and divide the species into two fermentation groups. The important substance in the identification of the organism is salicin. *B. œdematis maligni* ferments salicin, *B. chauvœi* does not ferment salicin but does attack saccharose.

Glucose broth and media containing salicin, especially when combined with serum, can be used for early rapid growth during isolation, but all readily fermented media may defeat the worker's intention unless the organism is rapidly subcultured. Milk in the case of *B. œdematis maligni* is a test substance of use in distinguishing the organism from others rather than a good medium for growth. Growing cultures are best maintained in chopped-meat medium or brain brew or in casein broth with a piece of egg-white. These are also the best media for preserving the vitality of resting cultures. In starting growth after a prolonged period of quiescence in a sealed tube, chopped meat, liver meat or chopped meat with glucose broth added are very suitable; subcultures into plain chopped-meat medium should be carried out in 48 hours and further work done from them.

B. œdematis maligni retains its pathogenicity with very little alteration through many years of subculture upon artificial media. The reaction of the medium, which should correspond to about pH 7·6, and the age of the culture influence the pathogenicity upon injection into experimental animals. Different strains show relatively little variation in pathogenicity under uniform and favourable conditions of growth.

Agglutinins are formed upon intravenous injection of washed suspensions of the bacilli into rabbits. The species can be divided into serological groups on the basis of the agglutination reaction (see p. 284).

B. CHAUVŒI.

B. chauvœi Arloing, Cornevin and Thomas, 1887. (Synonyms: *B. carbonis* Migula, 1895; *B. anthracis symptomatici* Kruse, 1896; Rauschbrand Bacillus of Foth in German literature.)

This organism is responsible for the disease of blackleg in cattle. It has often been confused with *B. œdematis maligni*, probably because clinical blackleg can be produced by both organisms. There are also certain points of resemblance in the characters of the two species; they are, however, quite distinct entities.

Motility. *B. chauvœi* is motile in young cultures and in exudates taken immediately from experimentally infected animals. The motility is variable or is lost in slightly older cultures.

Morphology. The rods in a young growing culture are about 3 to 4μ in length and 0·4 or 0·5μ in width. Spores are readily formed and are subterminal or central, they are more slender and elongated than in *B. œdematis maligni.* Navicular and club-shaped forms are produced in the presence of serum, or in media with fresh tissue, but they may also sometimes be seen in meat cultures: the navicular types are generally narrower and longer than in *B. œdematis maligni.*

B. chauvœi is Gram-positive in young cultures, but Gram-negative individuals are frequently present in older material.

Anaerobiosis. *B. chauvœi* is a very strict anaerobe.

Colony. In deep agar the colonies are generally round or lenticular; they may show outgrowths from the general mass. In surface growth they are round or may have fern-like processes; they are never woolly, and do not penetrate downwards into the medium.

Cultural reactions.

Meat medium. Gas is formed, the colour becomes pink and may fade later upon standing in the air, the medium is not blackened and there is no putrid odour.

Milk medium. Acid is formed and there is gradual clotting in about 3 to 6 days. Some gas may be evolved but there is no 'stormy' fermentation.

Coagulated serum is not liquefied.

Gelatin is liquefied.

Substances fermented. Glucose, galactose, lævulose, maltose, saccharose and lactose.

Substances not fermented. Glycerin, mannitol, dulcitol, inulin and salicin.

The substances in the above list important for the diagnosis of the species are saccharose and salicin. *B. chauvœi* does not ferment salicin and does ferment saccharose. *B. œdematis maligni* ferments salicin; in the writer's experience it does not ferment saccharose, but as stated above some strains apparently do. The distinctive point here is the failure of *B. chauvœi* to alter salicin.

B. chauvœi may upon some occasions grow rather feebly, and glucose may be added to the meat medium with beneficial results, but as always with glucose the subculture should be rapid. Liver-meat medium gives excellent growth. Meat medium and casein digest broth with a piece of coagulated egg-white are the best media for the preservation of cultures in the resting state. This organism is very pathogenic for mice and guinea-pigs; rabbits offer a marked resistance to infection. The pathogenicity is retained through long periods of culture under laboratory conditions.

The serological reactions are a further means of distinguishing *B. chauvœi* from *B. œdematis maligni* (p. 284).

B. NOVYI.

B. novyi Migula, 1900. (Synonyms: *B. œdematis thermophilus* Kruse, 1896; *B. œdematiens* Weinberg and Séguin, 1915).

Motility. *B. novyi* is motile in young cultures examined under anaerobic conditions in a sealed capillary tube. On a cold stage under the coverslip the rods are not seen to move.

Morphology. *B. novyi* is a stout Gram-positive rod 0·8 to 1μ thick and of varying length, often curved. The morphology should be studied in very young cultures, as autolysis occurs as early as the 14th to 16th hour of growth. There is a little pleomorphism in the forms adopted by *B. novyi*, and short chains and filaments, also granular forms, may be seen. Spores are formed in all media and may be subterminal or central; they are large and oval with slightly flattened ends. They are able to resist heating for a remarkable length of time, and will survive exposure to 97 to 100° C. for more than 20 minutes.

Anaerobiosis. Surface growths can only be obtained under conditions of strict anaerobiosis.

Colony. The colonies on plates are flat and tend to run into each other or spread over the surface in a delicate film. In agar shakes the most usual types of colony are delicate irregular snow-flake growths, but colonies resembling the conventional bursting grenade are sometimes formed.

Cultural reactions.

Meat medium. Gas is formed, a pinkish colour is produced which fades quickly, but in some samples of meat the colour shows little change.

Milk medium. After 10 to 30 days' incubation there is an acid reaction and clotting. The acid may sometimes appear earlier in vigorous cultures, but the clot is always delayed.

Coagulated serum. The medium is not altered.

Broth. Flocculi are formed quite early during the first hours of growth later these sink down towards the bottom of the tube forming a semi-opaque cloud after 18 to 36 hours.

Gelatin. The medium is liquefied. In the writer's experience the production of acid is feeble.

Substances fermented. Glucose, lævulose, maltose.

Substances not fermented. Glycerin, galactose, saccharose, lactose, dulcitol, inulin, salicin.

There is considerable variation of opinion in regard to the fermentation reactions. For instance, Kahn (1924) finds that his cultures regularly ferment glucose, lævulose, galactose, saccharose, lactose, raffinose, mannose and starch, while inulin and xylose are feebly attacked with a variable degree of fermentation. This is in agreement with Henry (1916). Wolf (1919–20) got no fermentation of galactose, saccharose or lactose, and Hall (1922) found that glycerin and glucose were fermented, while lactose, saccharose, salicin and inulin were not attacked.

B. novyi is of great importance in gas gangrene owing to the acute toxæmia it produces. It is less frequently found than *B. welchii*, but its incidence is none the less of very serious consequence. It is one of the organisms which also infects animals, producing a fatal œdema, with symptoms resembling those of blackleg (Kerry, 1894; Heller, 1920). It is interesting to note that Novy actually discovered the organism in the first instance accidentally by injecting a milk nuclein preparation made from fresh milk into guinea-pigs.

B. novyi is pathogenic for laboratory animals, and, in the writer's experience, cultures properly preserved retain this character indefinitely. Its role in the diseases produced by anaerobes is perhaps not yet fully recognized. It produces a soluble toxin of high potency (p. 268).

B. FALLAX.

This organism was discovered and first isolated by Weinberg and Séguin (1915) from infected wounds. It is an organism of some importance in gas gangrene; it is sometimes found, like other anaerobes, in the blood stream in early stages of gangrene or of massive infection of a large wound, but it may also be the chief anaerobe in a fulminating case of fatal gangrene and be present in the blood as a true invading organism. Two such cases were reported by Stokes (1916) and the cultures were passed on to the writer. It has also been recovered from an infected knee joint by Mutch and Mutch (1927), and it is reported in five cases of appendicitis by Weinberg, Renard and Davesne (1926). Some strains are pathogenic for guinea-pigs immediately upon isolation (Weinberg and Séguin, 1918), but lose this character very quickly; nevertheless, a strain derived by blood culture from a fatal gangrene, which had lost its pathogenic properties, produced fatal infections in mice when inoculated with a small dose of calcium chloride (experiment carried out by Gye in 1919).

Motility. The rods are motile in young cultures.

Morphology. B. fallax is a rather slender organism of about 3 to 6μ in length with rounded ends. It has a marked tendency to become Gram-negative, although quite young bacilli do retain the stain. The rods are very often curved. Spores are not formed readily upon any medium, but do occur in meat and upon coagulated serum. They are oval and generally subterminal.

Colony. On surface plates the colonies are round or crenated and may be granular. In deep agar shakes they are lenticular or bean-shaped or irregular but not woolly or flaked.

Cultural reactions.

Meat medium. Gas is formed and a pinkish colour is produced, there is no digestion and the medium is not blackened.

Milk. There is a slow clotting with production of acid and some gas in 3 to 7 days.

Coagulated serum. The medium is not liquefied.

Gelatin. There is no liquefaction.

There is some difference of opinion about the fermentation reactions of *B. fallax*. Henry (1916) considered that glucose, lævulose, maltose, galactose, saccharose, starch, inulin, and salicin were all fermented. Kendall, Day and Walker (1922) and Kahn (1922) found that it ferments glucose, lævulose, galactose, saccharose, lactose, and xylose; mannose is feebly attacked by some strains and not by others: glycerin, inulin and salicin, they found, are not attacked. In the writer's experience glucose, lævulose and maltose are fermented and some strains attack galactose.

B. fallax has rather a feeble type of growth and may easily be lost; it is best preserved in alkaline egg-fluid or upon the chopped-meat medium. Resting cultures will germinate after many years—nine in the writer's actual experience—but old cultures are less certain to retain their viability than most of the other members of the group.

A soluble toxin is formed (p. 272).

CERTAIN ORGANISMS OF RARE OCCURRENCE.

Certain organisms of rare occurrence are noted very briefly, and all the facts concerning them are dealt with in this place. They have been described in recent literature and belong either in the truly pathogenic group or in the semi-pathogenic division of the anaerobes.

An Organism isolated from an Acute Case of Appendicitis.

An organism was described by Ukil (1922) isolated from an acute case of appendicitis. It is a Gram-positive rod 2 to 8μ in length. The spores which are formed very readily are subterminal; they resist boiling for 4 minutes. The organism is non-motile and without flagella. It is a strict anaerobe. It is pathogenic for mice and guinea-pigs when injected in doses of 5 c.cm. It produces a local œdematous swelling of considerable extent accompanied by petechial hæmorrhages on the surfaces of the muscles and a gelatinous œdema, which is sometimes white and sometimes red in colour. There is no infiltration of gas. The infection is not as a rule fatal, and even very extensive lesions disappear gradually in the course of 3 to 4 days. Similar lesions are evoked in rabbits and mice. A toxin is evolved and the filtered products injected in the dose of 8 c.cms. calls forth a pink œdema of the whole abdominal wall. Normal horse serum and the antisera of the Pasteur Institute against *B. welchii*, *B. novyi* (*B. œdematiens*) and *B. œdematis maligni* (vibrion septique) do not neutralize it. The histological study of the appendix showed the presence of the organism in the mucosa, the submucosa and the subperitoneal blood vessels, which were markedly congested.

So far this organism stands alone, but the account of it is sufficiently complete to make it intelligible and open to confirmation and criticism by other workers. It is of some slight importance as being another anaerobe isolated from the human intestinal tract, which, while not apparently acutely pathogenic when isolated upon artificial media, nevertheless produces a toxin resembling in nature that of the acutely pathogenic forms. In position it approximates to *B. novyi*.

A Toxic Organism from Gas Gangrene and Appendicitis.

In 1922, Sordelli (1922[1 & 2]) noted an anaerobe which he isolated from a case of gas gangrene and later from a case of appendicitis. It is highly pathogenic for guinea-pigs and rabbits. A soluble toxin is produced which resembles that of *B. novyi*, from which, however, it is distinct. The toxin of Sordelli's organism is not neutralized by the antisera against *B. welchii*, *B. novyi*, *B. œdematis maligni* and *B. histolyticus* made by the Pasteur Institute. The author makes an antiserum in horses by the injection of the soluble toxin, and this serum, while it neutralizes the toxin of the homologous organism and protects against infection with the living culture, has no inhibitory effect against *B. welchii*, *B. œdematis maligni*, *B. novyi* or *B. histolyticus*.

Sordelli gives further details (1923) and suggests the name *B. œdematis sporogenes*. Hall and Scott (1927) confirmed many of his findings. They proposed the name *B. sordelli* for the organism to avoid the inevitable confusion that would be caused by Sordelli's title. This bacillus should be classed as allied to *B. novyi*. It is described, however, as having greater proteolytic capacity and is apparently specifically distinct.

B. œdematoides *Meleney, Humphreys and Carp*, 1927.

This name is suggested for an apparently new pathogenic anaerobe isolated from a fatal infection of an operation wound. It is a Gram-positive rod 1 to 4μ in length and motile in young cultures. Spores are formed in all media except those containing dextrose. In deep agar the colonies are small and discrete and described as mossy. On blood agar plates the organism is only very slightly hæmolytic. The odour is cheesy but not suggestive of butyric acid. Gelatin is liquefied, a very slight pitting of coagulated serum is recorded but no actual liquefaction, casein of milk shows slight digestion, and meat may be very slightly blackened.

The fermentation capacity is poor. Milk is only rendered acid after prolonged incubation. Acid and gas are formed in dextrose, but lactose, saccharose, salicin, mannitol and glycerin are not fermented. The organism is pathogenic for laboratory animals, producing a picture closely resembling that produced by *B. novyi*. An exotoxin is evolved which is filterable, thermolabile, has a latent period and is capable of evoking an antitoxin when injected into rabbits either subcutaneously or intravenously. The immune body is specific as is the toxin. The organism is serologically distinct from *B. novyi* and *B. œdematis maligni*. Three strains were isolated, one from the lesion of the human case and two from 2 out of 4 tubes of catgut used in the hospital.

B. histolyticus.

B. histolyticus Weinberg and Séguin (1915 and 1916[1]). This organism was discovered by Weinberg and Séguin in wounds and they showed that it was implicated in gas gangrene and in serious local lesions although not actually found as causing an acute gangrene by itself. It has since then been found in earth in various parts of the world (Peterson and Hall, 1923); it has been isolated from fæces and also from cases of appendicitis (Weinberg, Renard and Davesne, 1926). It is not an easy organism to isolate and has probably often escaped detection. Torrey (1924–5) isolated *B. histolyticus* from the ileo-cæcal region of the intestine of man upon two occasions. The material was obtained at operation.

Morphology. *B. histolyticus* is a motile rod often arranged in pairs. It is about 0·5 to 0·8μ broad and about 3 to 5μ in length. The bacilli degenerate very soon in cultures and after 24 to 48 hours' growth many Gram-negative individuals may be seen together with fusiform and skittle-shaped involution types. The spores which are readily formed upon all media are oval and usually subterminal; they are considerably wider than the rod in which they arise.

Cultural reactions. *B. histolyticus* is not a very strict anaerobe and some strains are definitely capable of some degree of growth on agar slopes under aerobic conditions. There seems to be a progressive adaptation in the direction of oxygen tolerance, as a type culture obtained from Dr. Weinberg in 1918 was then a stricter anaerobe than it was found to be in 1926.

Colony. The surface colonies are delicate and flat; they have crenated or irregular edges. Colonies in deep agar shakes are arborescent or coral-like with fine woolly ends to the branches. The organism is hæmolytic.

Meat medium. The medium is digested with the production of a white crystalline deposit, considered to be tyrosin. There is not much evolution of gas and the odour is not putrid.

Milk. The medium is digested after the formation of a soft clot.

Coagulated serum is liquefied.

There is a good deal of argument about the fermentations produced by this organism; it appears that in pure culture it is very inert in sugar media. Some authorities (Henry, 1916) claim that it ferments glucose, lævulose and maltose; others (Kahn, 1924; Kendall, Day and Walker, 1922) consider it to be a non-fermentative organism. A soluble toxin is produced to which Weinberg and Séguin were able to obtain an antiserum by injecting horses with graduated doses of the toxin. This species has a remarkable capacity for the digestion of tissue *in vivo* (see p. 262).

B. sporogenes.

B. sporogenes Metchnikoff, 1908. (*B. putrificus verrucosus* Zeissler, 1923; this author gives as a synonym *Paraplectrum fœtidum* Weigmann.) *B. sporogenes* and the sub-groups that are identified as *B. parasporogenes* and *B. centrosporogenes* are of great importance in every kind of work upon anaerobic bacteria. This species is present in practically all the local lesions in gas gangrene cases and in all wounds heavily infected with anaerobes; it can be carried into the blood stream and recovered from that situation during life. It is universally present in the intestine of man and animals, and in earth in cultivated areas and wherever there is grazing of stock or wild animals. It is found as a putrefying organism in decaying cadavers and in fermenting vegetable matter. In gas gangrene, as will be seen later, apart from its local action, it is actually concerned in the production of mixed or putrefying gangrene, although acting alone and without the presence of one of the pathogenic forms already described it is incapable of setting up a generalized infection.

B. sporogenes is remarkably resistant to heat and to other adverse circumstances, and it is the most frequent contaminator of all other anaerobe cultures. It is capable of very close microbic association with all other anaerobes, and it exhibits very remarkable talents for latent survival under unsuitable conditions and for germination after long periods of delay. It has, in addition, a strong tendency to grow in mixed colonies. It is relatively easy to isolate in pure culture from wounds but less so from sewage, fæces or other mixed material.

Apart from *B. parasporogenes* and *B. centrosporogenes* (to be referred to later on) *B. sporogenes* itself is a species capable of subdivision on the agglutination reaction. As in other similar cases the writer holds that it is advisable to keep to the rather wide interpretation of species rather than to multiply names upon distinctions that are not very obvious. The species has a cultural solidity that identifies it clearly and seems to serve both scientific and practical purposes of classification.

Morphology. *B. sporogenes* is an actively motile rod of about 3 to 7μ in length. Spores are very readily formed in all media in pure cultures, they are oval in shape and central or subterminal in position. It is a curious and somewhat important point to note that in mixed symbiotic cultures spore formation is much less obvious. The range of bacillary forms is more restricted than in the pathogenic anaerobes, but long filaments may be produced upon many different media, particularly in chopped meat and coagulated serum or egg. The bacilli are Gram-positive, but in old cultures Gram-negative individuals are found.

Cultural reactions. *B. sporogenes* is less exacting in the matter of anaerobiosis than some of the strictest forms; nevertheless, here, as indeed in all anaerobes, the activity of fermentation and proteolysis depends somewhat upon a suitable and sufficient degree of anaerobiosis.

Colony. There is a good deal of variation in the colonies but in general there is a tendency to form woolly colonies with tangled filaments. On the surface they penetrate into the agar if incubated for long periods, but they may also grow as transparent snake-like wisps or in irregular transparent droplets; later, most colonies become opaque and thickened towards the centre, some are yellowish in colour. Deep colonies are woolly as a rule.

Meat medium. There is a vigorous growth; gas and an alkaline reaction are produced; the medium is digested and blackened to some extent. The nature of the change of colour varies with different samples of the medium: in some cases there may be no actual blackening, although active digestion is going on; the colour in this case may be a dark brick-red. The addition of iron filings or a nail produces an intense black colour; the odour is putrid.

Milk. Digestion occurs with or without a certain amount of clotting, and the reaction becomes alkaline.

Coagulated serum. This medium is liquefied and may become darker in colour.

Alkaline egg broth. There is formed a flocculent precipitate or even a soft clot which sinks to the bottom of the tube leaving a clear supernatant fluid; the clot is digested to a greater or less extent.

Gelatin. The medium is liquefied.

Glucose, lævulose and maltose are fermented.

B. sporogenes, as will be seen from the above, is an actively proteolytic organism.

B. parasporogenes *McIntosh and Fildes,* 1917.

This organism agrees with *B. sporogenes* in all its characters except in the type of colony produced and in the agglutinins formed. The colony is constantly smooth and round in surface growths and lenticular or slightly irregular but never woolly in deep agar shakes. The importance of this type of colony in so contaminative an organism as this merits particular attention, and the name *B. parasporogenes* is used to distinguish it.

B. centrosporogenes.

B. centrosporogenes is a type described by Hall (1922). It is a large Gram-positive rod occurring singly, in twos and threes, in short chains, and in filaments. Spores are formed early, especially in media devoid of sugar; they are central and do not swell the rod. The bacilli are highly motile in young cultures and non-motile in old ones. Colonies on blood agar are hæmolytic transparent dewdrops, later yellowish and more opaque, growing finally to 1 to 2 mm. if well separated. The organism is proteolytic, digesting coagulated egg and meat medium with a tendency to blackening. Its right to specific rank is rather doubtful; its distinction from *B. bifermentans* seems to rest on the motility alone; no agglutination reactions are given.

B. bifermentans *Tissier and Martelly*, 1902.

This organism was first described by Tissier and Martelly in 1902 under the name of *B. bifermentans sporogenes*. It was called bifermentans owing to its being the first anaerobe in which a capacity to split both sugar and protein was definitely recognized. It is occasionally found in cases of acute gangrene as well as in late septic wounds uncomplicated by gangrene. It is also present in the intestine of man (Kahn, 1924).

Morphology. In the non-sporing state it is a stout rod closely resembling *B. welchii*. Individual elements may be very short and chains are frequently formed. Spores occur in all media; they are usually central but may also be subterminal.

Cultural reactions. Surface colonies are round or crenated. Deep colonies are lenticular or irregular, but without woolly outgrowths.

Meat medium. This is blackened and digested with the evolution of gas and a putrid odour.

Milk medium. Casein is precipitated and later digested.

Coagulated serum. Liquefied.

Gelatin. The medium is liquefied.

Alkaline egg broth. A soft clot is formed which is later digested. Specific agglutinins can be produced in rabbits inoculated intravenously with the organism.

B. aerofœtidus *Weinberg and Seguin*, 1916.

Morphology. *B. aerofœtidus* is a feebly motile small bacillus about 2 to 4μ in length with a tendency to be Gram-negative.

Growth reactions. Spores are not readily formed upon any medium; they are subterminal. Surface colonies on serum agar or blood agar are round and transparent and may attain to 1 or 2 mm. in diameter after 24 hours' incubation. Deep colonies are small and irregular. The organism is not hæmolytic.

Meat medium. A putrid odour is evolved, the meat changes colour, first reddening and then blackening, the organism is definitely proteolytic but much less actively so the *B. sporogenes*.

Milk medium. Clot and gas are formed in 24 to 48 hours; later there is a certain amount of digestion.

Coagulated serum. There is liquefaction, and a putrid odour is produced.

Gelatin. This medium is liquefied.

Alkaline egg broth. A soft clot is formed which may be partially digested.

According to Henry (1916), glucose, maltose, lactose, lævulose and salicin are fermented.

Kahn (1924) states that glucose, maltose, lactose, laevulose, galactose and mannitol and xylose are fermented while salicin gives a variable reaction.

B. aerofœtidus was considered to be feebly pathogenic immediately after isolation by Weinberg and Séguin, but this has not been confirmed by other workers. It is found in cases of gangrene and in wound infections, and has been recovered from the alimentary tract of man by Kahn.

B. tertius.

B. tertius Henry, 1916. (Synonyms: Rodella's Bacillus III; von Hibler's Bacillus IX; Zeissler considers that *B. tertius* is a synonym of *B. amylobacter* van Tieghem.)

Morphology. *B. tertius* is sluggishly motile in quite young cultures. The rods are slender and Gram-positive when young (12 to 18 hours' growth), but soon tend to become Gram-negative, especially in fluid media.

Spores are readily formed; they are oval and strictly terminal.

Growth reactions. *B. tertius* requires only a moderate degree of anaerobiosis. In this connection *B. aero-tertius* of Fleming (1919) should be considered, which will grow both aerobically and anaerobically and was recovered from wounds; its cultural characters are those of *B. tertius*.

Surface colonies are small (1 mm.) and are round or crenated. They are iridescent and transparent when young, but become more opaque and slightly granular on certain media when older. In deep shakes they are small and lenticular or irregular in shape and do not show branching filaments.

Meat medium. There is no blackening and no digestion of the medium. A pink colour is produced and gas is evolved.

Milk medium. Acid is produced and some degree of clot in 3 to 6 days with evolution of gas. The growth in milk may be poor.

Coagulated serum. The medium is not liquefied.

Gelatin. This is not liquefied. Kahn (1924) has claimed that gelatin is finally liquefied upon long incubation. In the opinion of the writer this does not occur in perfectly pure cultures. Kahn does not get the liquefaction until after 30 days' incubation. Kendall, Day and Walker (1922) are in agreement with the writer and do not find liquefaction of gelatin.

The species is highly saccharolytic; glucose, lævulose, galactose, maltose, saccharose, lactose, mannitol and salicin are fermented. Glycerin, inulin and dulcitol are not fermented. Hæmolytic substances are elaborated by *B. tertius* (Kahn, 1924).

This organism is very frequently present in wounds and in the alimentary canal of man and animals. It may appear as early as the first week in life in breast-fed infants (Hall and Matsumura, 1924). *B. tertius* is non-pathogenic for laboratory animals in pure culture.

B. multifermentans tenalbus *Stoddard,* 1919.

This is a non-pathogenic organism recovered by Stoddard from a case of gas gangrene. It was infrequent in wounds, but has a certain importance because of its morphological resemblance to *B. œdematis maligni.* It has lately been found contaminating chocolate creams in America (Hill, 1925).

Morphology. B. multifermentans tenalbus is a Gram-positive motile bacillus. The spores are central or subterminal. When grown upon egg or serum media navicular types and filaments and swollen club-shaped individuals are formed.

Growth reactions. Surface colonies under anaerobic conditions may grow to a large size (3 to 5 mm.), they are round, with somewhat irregular edges. In glucose agar shakes the colonies are white and opaque, lenticular in shape or with irregular outgrowths.

Meat medium. Gas is formed in this medium ; there is no obvious digestion and no blackening ; the odour is not putrid.

Milk medium. Acid and clot are produced.

Coagulated serum. The medium is unchanged.

Gelatin. There is no liquefaction of the medium.

Glycerin, maltose, lactose, raffinose, glucose, saccharose, inulin and salicin are fermented. Mannitol and dulcitol are not fermented.

Biochemical Reactions.

FERMENTATIONS, PROTEOLYSIS AND SPLITTING OF FATS.

The biochemical reactions of the anaerobes have been the subject of a great deal of work, and numerous careful analyses have been made of the growth products. (Wolf and Telfer, 1917; Wolf, 1918-19 and 1919-20; Wolf and Harris, 1917 ; Kendall, Day and Walker, 1922 ; Brown, 1921, 1922, 1924 and 1925 ; Hall and Randall, 1922 ; Bushnell, 1922 ; and Wagner, Meyer and Dozier, 1925.)

Proteolysis. The proteolytic or saccharolytic type of metabolism affords, as has been stated above, a rough biological series of differentiations. All bacteria are proteolytic in a certain degree, but as used in this connection proteolysis implies the capacity to attack higher protein substances, such as gelatin, casein, serum, egg-white and fibrin. The classification of the anaerobes in regard to an ascending capacity for proteolysis would lead to the following sequence (Brown, 1925) : *B. fallax, B. multifermentans tenalbus* and *B. tertius* are the least proteolytic ; they attack none of the proteins mentioned. *B. chauvœi, B. œdematis maligni* and *B. novyi* are the next lowest, attacking gelatin but neither casein nor serum. Then comes *B. welchii*, which, in addition to liquefying gelatin, has a very slight capacity for digesting casein, but is unable to attack egg-white. The faculty for splitting casein is disputed by Kahn (1924), also by Kendall, Day and Walker (1922), and is in any case below the threshold of macroscopic observation. *B. sporogenes, B. histolyticus, B. bifermentans* and *B. aerofœtidus* attack all the substances already mentioned, including fibrin.

This ascending scale of proteolytic activity seems, in spite of a few detailed points still under dispute, to be considered valid in its main outlines by all the biochemical workers. It is the sequence that is recognized also in the mere inspection of the macroscopic growth characters. The actual chemical action is agreed upon in most cases, but there is some divergence of opinion upon the nature of the liquefaction of gelatin. Kendall, Day and Walker (1922) consider that there are two different processes, namely, that found in the case of *B. welchii* and the less proteolytic forms, where a soluble gelatinase is not demonstrable, and that occurring in the highly proteolytic organisms such as *B. sporogenes*, where an active proteolytic enzyme is evident. Brown (1925) seems to consider the liquefaction of gelatin as being always a true proteolytic process in these organisms. The attack on the higher proteins by the more proteolytic organisms results in the breaking down of nitrogen into ammonia and amino-acid nitrogen. Hydrogen sulphide is also produced. Wolf 1918-19[2]) shows that in chopped-meat medium containing 0·87 per cent. of nitrogen, 71·5 per cent. will be changed into ammonia and amino-acid nitrogen in 286 hours.

Action on carbohydrate. The anaerobes exercise a very definite action on various sugars, fermenting large quantities in the case of the more saccharolytic types with the evolution of considerable amounts of gas. The gas is carbon dioxide and hydrogen. *B. welchii*, according to Wolf (1918-19[2]) in one of his analyses, broke down 13·8 gm. of lactose per litre of milk with the evolution of 2,714 c.cm. of gas. In the case of this organism the effect of hydrogen-ion concentration was examined, and it was found that metabolism ceased at pH 4·82 in glucose peptone medium. Wolf also ascertained that in a medium of a given initial reaction the final reaction at which growth ceases is constant for each organism. The point at which growth is limited is at an acidity approximating that of 1/30,000 N solution of hydrochloric or sulphuric acid. *B. welchii*, according to Humphreys (1924), in the fermentation of glycerin produces the aldehyde acreolin in sufficient quantities after 18 to 24 hours' incubation to inhibit growth and kill the organism. In the fermentations set up by the anaerobes, ammonia may be produced at the same time as acids. This maintains the culture in an alkaline balance sufficient for survival, but where the medium is rich in carbohydrate, and no great quantity of ammonia is produced, death supervenes from the increase in acidity.

Action on other substances. Brown (1925) noted the capacity of the anaerobes to attack the fat of cows' milk and to hydrolyze it into soaps and fatty acids. *B. welchii, B. chauvœi, B. œdematis maligni, B. histolyticus* and *B. sporogenes* were all shown to hydrolyze milk fats. *B. novyi* and *B. fallax* were not tested for this character.

Toxins and poisonous growth products. As a result of growth, soluble bacterial toxins are produced and certain other poisonous products are also developed. The toxins will be considered below (p. 261). The other poisonous products are non-specific and are not antigens. They have not been very closely studied. It is probable that most anaerobes produce these substances in some degree and that the harmful action may be due to different constituents in the case of different anaerobes.

B. sporogenes grown in meat medium produces one of these poisons, it is heat-stable and passes over from the medium upon distillation. Barger and Dale (1915) were of the opinion that it was a volatile poison probably of the nature of an ammonia base. McIntosh and Fildes (1917) considered that it was a sulphide, probably ammonium sulphide. It produces death in guinea-pigs in doses of 1 to 4 c.cm. injected intraperitoneally. Another of these non-specific bacterial growth products has been described recently by Kendall and Schmitt (1926) from cultures of *B. welchii*. It is resistant to heat and is distinguished from butyric acid. It appears only in media with fermentable carbohydrate. The authors are of the opinion that the organism produces carboxylase from the utilization of carbohydrates, and this in turn decarboxylizes the amino-acid, splitting off CO_2 and leaving the primary amine behind. They consider that the substance has an action similar to that of histamine. It causes contracture of the isolated uterus and small intestine of guinea-pigs, and abrupt but transitory fall in blood pressure upon intravenous injection in the dog under ether anæsthesia. The substance was produced in 66 out of 72 *B. welchii* cultures tested.

Wassermann and Ficker (1922), Kojima (1922 and 1923[4]) should also be consulted for information upon this aspect of the growth products of *B. welchii*: they were the first authors to consider their clinical bearing.

(For reduction and respiration see Vol. I.)

Distribution and Persistence.

The anaerobes of gas gangrene are widely distributed in nature. They seem primarily to be inhabitants of the intestinal tract of man and animals. All the anaerobes dealt with in this chapter have been isolated from the intestine of man with the exception of *B. chauvœi* and *B. multifermentans tenalbus* (Kahn, 1924). They are also present as putrefying organisms in decaying vegetable and animal matter. The formation of resistant spores permits of their long survival in earth and they are widely disseminated in dust.

The viability of anaerobic bacteria must be considered under two aspects, namely in relation to the capacity for continued growth of cultures in an active state, and secondly in connection with the resistance of spores to adverse conditions. In regard to growth capacity certain anaerobes are delicate and may succumb to adverse conditions very readily. These are mostly those types which delay to form spores or which only form spores under certain circumstances. Species liable to show poor vitality are *B. fallax, B. aerofœtidus, B. novyi* and *B. chauvœi*; this is manifested by failure to grow or by feeble growth, by delay in sporulating and by the extinction of the culture through the accumulation of its own growth products. *B. welchii*, although one of the most vigorous of all anaerobes, must be added to the list of those organisms whose growth may culminate in their own destruction. The conditions under which this occurs have been referred to above (p. 235).

The resistance of the anaerobes to heat is in general very great. *B. novyi*, for example, derived direct from wound exudate, will survive exposure to a temperature of 99–100° C. in broth for 15 minutes. No very accurate work has been done on the heat survival of spores. A very high degree of resistance is also shown to the action of disinfectants. Schütze (1915) found that *B. sporogenes* spores would germinate after 8 days in 5 per cent. solution of phenol. This great capacity for survival is of practical importance, and care must be taken to exclude the spores of these organisms from solutions injected intramuscularly and subcutaneously, and from instruments, particularly syringe needles. Wanke (1926) reports two cases of infection with *B. welchii* after the injection of morphia, and the source was traced to the needles used, which were kept in 96 per cent. alcohol. The organism was grown from the needles and the cultures were tested for pathogenicity in animals. Lande (1926) has three cases arising from the injection of camphor oil, caffein and asthmolysin. Fraenkel and Wohlwill (1922) describe three instances occurring in the same hospital from the injection of adrenaline, caffein and camphor, the three injections being given on the same evening. All three cases were fatal. The data were carefully documented and there was an investigation of the solutions and needles, which, however, yielded negative results. The gangrenes developed at the site of the injections. Kemkes (1923) reports a case of fatal gas gangrene with death in 36 hours from injection of novocain into the sciatic nerve.

Pathogenic Action.

GENERAL NATURE OF THE PATHOGENIC ACTION OF THE ANAEROBES OF THE GAS GANGRENE GROUP.

The pathogenic action of the anaerobes in established disease depends upon the absorption of true toxins, perhaps also in some cases upon the auxiliary effect of non-specific bacterial growth products and upon the actual invasion of the tissues of the host by the organism. These effects may be accumulated into one syndrome or may be produced separately, according to the nature of the disease. Thus *B. œdematis maligni* may produce a fulminating gangrene in man, in which there is a massive invasion of tissue primarily at the site of the injury, and invasion of various organs, notably the liver. At the same time the toxin elaborated by the bacteria exercises a profound effect and is probably the actual cause of death. In the disease of sheep known as braxy, also caused by *B. œdematis maligni*, the invasion occurs from the alimentary canal, and a small lesion in the stomach is the site of entry; the disease is here both invasive and toxic, but the toxic element appears to predominate.

B. welchii, the most invasive of all the anaerobes when entry is obtained by means of a wound may, nevertheless, set up types of disease which are chiefly toxic. These divergent manifestations produced by one species are merely special examples of the pathogenic capacity of the type, and are inherent in its biology. The divergence in the character of the disease is due to the particular relation in which the bacterial agent stands to the body, and not to any real difference in its action.

In gas gangrene, and indeed, in almost any aspect of the relation of these anaerobes to disease, it is found that the group may be divided into three sections: (i) The acutely pathogenic organisms comprising *B. welchii*, *B. œdematis maligni* and *B. novyi*; also *B. chauvœi* in animals. (ii) The organisms pathogenic in a lesser degree, namely *B. fallax* and *B. histolyticus*. These two species are both toxic and invasive, and *B. histolyticus* has in addition a remarkable power of local digestion of the living tissues. (iii) The organisms which have no appreciable pathogenic action in pure culture, but may, nevertheless, exercise a harmful effect in the presence of each other and of the more pathogenic types. To this group belong *B. sporogenes*, *B. centrosporogenes*, *B. parasporogenes*, *B. aerofœtidus*, *B. tertius*, *B. bifermentans* and *B. multifermentans tenalbus*, and in addition, *B. cochlearius*, *B. tetanomorphus* and the round end-sporing bacilli considered in Chapter X. All these types are important in producing a nidus in which the pathogenic forms may proliferate and elaborate their toxins.

The bacterial associations in acute gangrene may consist of two or more pathogenic forms or of almost any combination of pathogenic with non-pathogenic. The purely saprophytic anaerobes may be carried into the blood stream and have the appearance of true invaders; no doubt they do at times proliferate to some extent beyond the actual site of the wound, but their presence is transitory and unimportant, unless accompanied by one of the pathogenic forms. In gangrenes and anaerobe infections arising otherwise than from wounds and injuries, the different species may again act independently or in association with each other, and with aerobic organisms. While the anaerobic infection of wounds presents this complex bacterial picture it is nevertheless true that the organisms can be dealt with by the body with great success, unless one (or more) of the pathogenic types gets the opportunity for attack as distinct from the mere licence to proliferate within the wound. Once the pathogenic type has gained entry, however, the disease may proceed with great rapidity.

The conditions that have been described above as arising from wounds may also arise in injury of various kinds, in the infection of surgical wounds invaded by anaerobes from the intestine, in septicæmias, in peritonitis, in lung gangrene, gangrenous bronchiectasis and putrid pleurisy, in gangrenous appendicitis and in puerperal infections. These last are often only recognized *post mortem*. It is easily understood how in civil practice, owing to the peculiar nature of their unimportant onset, without characteristic disturbance, these infections may in the more obscure cases be overlooked and the subsequent rapid intoxication be ascribed to other causes.

Before dealing with the characteristic experimental infections which afford both a description and an analysis of the natural infections in the case of each of the pathogenic anaerobes, some points of general importance should be considered.

In the case of the three pathogenic anaerobes of gangrene and also with *B. chauvœi*, infections can be set up in experimental animals by the inoculation of wound exudate or œdema fluid from cases of the disease. As, however, these fluids are rarely free from other anaerobes, the study of the pathogenic action is best made with material derived from pure cultures. The picture in experimental animals closely resembles that seen in natural infections, but in considering any particular case it should be remembered that the disease will show certain variations according to the site of attack. No inoculation made either with wound exudate or with pure untreated cultures is a repetition of a natural infection. This must be clearly understood. In experimental inoculations where the complete culture is used, actively growing organisms, preformed toxins and other bacterial growth products are also injected into the animal. In natural infections (apart from a small group where actively proliferating organisms gain access from the alimentary canal) the process is different. The organisms, usually in the form of spores, are admitted into the body in some way such as by the contamination of wounds, or by entry into the blood stream from ulcers in the alimentary tract, or by ingestion, as is now supposed to occur in blackleg. The production of one of the anaerobe diseases under natural conditions depends upon the proliferation of the bacteria in a position suitable for the elaboration of toxin, this breaks down the local resistance, and the multiplication becomes intense, with the production of larger amounts of toxin, and the interaction of these factors give the final picture of the disease.

In the case of wounds and injuries the conditions suitable for proliferation can be inaugurated in various ways; the injury may produce dead or devitalized tissue and the blood supply may be impaired so that growth starts in a favourable medium, the multiplication of the saprophytic organisms assisting upon occasion in the setting up of the primary local focus. (See also, on pp. 279-80, the action of local debilitants.) While the interaction of the factors will vary in individual cases the general mechanism is indicated above and obtains corroborative evidence from the fact that washed bacilli and spores of all the pathogenic anaerobes may be injected without apparent result into experimental animals.

It should be noted that experimental infections give an excellent account of gas gangrene, because the nature of the method of attack is similar and the general circumstances of the relation to the body are alike, but the pathogenic action of the anaerobes, as will be seen in the sequel, is not restricted to this type of disease.

Pathogenic Action of *B. welchii*.

The pathogenicity of *B. welchii* varies very greatly; some strains are extremely virulent, some are scarcely pathogenic at all, judged by the same experimental criteria. One strain will show variation in pathogenicity at different times, but this is to some extent due to the method of culture.

Guinea-pigs, mice and pigeons are all susceptible to infections with this organism. Rabbits may also be infected but are less susceptible. Cornell (1925[1 & 2]) has utilized this fact to produce chronic infections in these animals, which throw further light upon the pathogenic action of the organism.

If 0·2 to 1 c.cm. of a *B. welchii* culture grown in meat medium or in broth be injected intramuscularly into the thigh of a guinea-pig, an acute disease is produced resulting usually in death within 24 to 60 hours. (The material inoculated should be an actively growing young culture of 24 hours' incubation.) In the course of a few hours from the time of inoculation the animal becomes quiet, but continues to feed; there arises a marked swelling of the limb injected and there may be crepitation from the formation of gas. The œdema spreads all over the abdomen and into the axillary region, the extent of the œdema depending upon the virulence and rapidity of the progress of the disease. The skin may be friable, and in some cases the œdema fluid escapes through the formation of a perforating necrosis: if this occurs the animal may recover; sublethal doses produce lesions of greater or less severity which are not fatal.

Post-mortem appearances. Examination immediately after death shows an extensive fluid œdema usually slightly blood-stained, but never to the same extent as in an infection with *B. œdematis maligni*. There are often globules of fat floating in the œdema fluid. There may be some gas in the tissues; the muscles of the limb at the site of the injection are sodden and friable and have the appearance of being somewhat digested; they are of a pale pink colour. A sour acid odour is evolved, but there is no blackening, and there is no suggestion of putrefaction. The vessels of the mesentery and intestine may be injected, but the internal organs do not as a rule show any conspicuous macroscopic changes with the exception of the suprarenal glands. These may be of a deep red colour, especially if the death of the animal has taken place rapidly; in other cases they may be mottled or hardly coloured at all. If the animal survives till the third day the suprarenals do not as a rule show any change in colour.

In the disease as produced in mice the liver is small and pale in colour, the kidneys and suprarenals are congested and the bladder is sometimes full of blood-stained fluid. The duodenum is dilated and may be pink in colour. If animals are left for some time after death, particularly at higher temperatures (25 to 30° C.), the liver may show the characteristic foamy appearance, which has received more than its due share of attention as a post-mortem appearance. In all cases the blood stream is invaded at a relatively early stage of the disease.

Microscopic examination. Very large numbers of bacilli are to be found in the muscles at the site of inoculation and there are also considerable numbers in the exudate. Spores are not as a rule formed in the body of experimental animals during life. Short chains of 3 to 5 elements may be found on the peritoneal surfaces, but even after death long filaments are not produced. Kettle (1919) has given a clear description of some of the microscopic features in anaerobic infections and this account is taken from his work. The most striking characteristic and one common to infections with any of the pathogenic anaerobes of this group is the absence of cellular pyogenic response. The muscle fibres are quiescent and there is no accumulation of wandering cells ; in the subcutaneous and connective tissues, however, some degree of polymorphonuclear leucocytosis may be present. The behaviour of the leucocytes seems to depend upon the actual and immediate effect of the toxin, as in its absence or where its effect is weakened, phagocytosis takes place. There is a late necrosis of the blood vessels but no very characteristic early lesions. Thrombosis of veins and capillaries is frequent and there is clear evidence of a toxic destruction of red blood corpuscles. In advanced lesions, hæmorrhages and hæmolysis frequently occur. Kettle did not come to any clear conclusion as to the differences between the lesions caused by the three pathogenic anaerobes, except that *B. œdematis maligni* and *B. novyi* appear to give rise to more œdema and to greater vascular changes than *B. welchii* ; also hæmorrhage and thrombosis were, he thought, particularly associated with *B. œdematis maligni*.

The macroscopic appearances at post-mortem examination would support these suggestions. The characteristic lesion at the site of inoculation or at the site of the injury in all gas gangrene infections consists in the death of the cells, brought about by the action of the toxins. The muscles, nerves and the epidermis and its derivatives are affected first, but eventually the blood vessels and connective tissue are also attacked. Kettle did not find lesions selectively localized in voluntary muscle either in human or in experimental gangrene except those at the site of inoculation or of injury. In one case, however, he found a lesion in the heart muscle: numbers of bacilli were lying free among the desquamating fibres; the muscle fibres had lost their striations and the nuclei no longer took up the stain.

Liver. Local lesions appear frequently in the liver. Bacterial emboli are not formed, but the organisms are held up mechanically and small colonies are developed. The cells are killed in a wide zone round the colonies. The process appears to be extremely rapid and is in the nature of a coagulation necrosis.

Kidney. The lesions resemble those in the liver and are due to the presence of colonies of organisms, the changes are first seen in the cells of the convoluted tubules.

Lymphatic glands. The organisms reach the glands by permeation along the lymph vessels and give rise to the usual type of quiet necrosis, the lack of any cellular reaction is characteristic.

Nervous system. The peripheral nerves in the local lesions show necrotic changes similar to those in the muscles and other tissues. Lesions were not found in the central nervous system, but in one case there was a condition of cerebral meningitis apparently due to one of the anaerobic bacilli. The membrane was congested and covered with polynuclear leucocytic exudate, and there was active phagocytosis of the bacilli by the leucocytes. Many bacilli were present in the vessels underlying the cerebral cortex but the nerve cells showed no change. Similar, not very pronounced changes are recorded by van Gehuchten (1921) in guinea-pigs. The lesions are more definite in the cases that evolve slowly.

The changes due to toxin as such will be dealt with when the toxins are discussed (see p. 265).

A chronic infection has been produced in rabbits by Cornell (1925[1 & 2]). He inoculated *B. welchii* cultures (isolated originally from fæces) in small doses into the animals, either into the spleen under ether anæsthesia or subcutaneously. In successful cases a chronic disease lasting up to five months was produced. The condition was characterized by anaemia, convulsions and diarrhœa. At death there was sometimes, but not always, red staining of the skeletal muscles ; the spleen was small and paler than normal. The spinal cord in sections showed meningeal thickenings but no inflammatory reaction ; numerous bacilli, Gram-positive and of the morphology of *B. welchii*, were found in the meninges at all levels, but the cord itself was apparently normal. *B. welchii* could be cultured from the spleen, bone marrow and liver. Microscopically the most important change was in the bone marrow ; it showed greatly increased erythrocyte activity, the medullary fat cells being replaced to about half their number by active hæmopoietic structures. The cellular elements showed as many megaloblasts as normoblastic erythrocytes. The effect of these chronic infections upon the blood picture is interesting. Cornell finds a constant anæmia of varying degree characterized invariably by a marked anisocytosis. He considers this to be due to the direct effect of the toxin of *B. welchii*. His analysis of the action and his reasons for incriminating the toxin are dealt with on p. 266. Cornell could also produce these chronic infections by inoculating suspensions of bacilli grown on the surface of blood agar plates under anaerobic conditions.

PATHOGENIC ACTION OF *B. ŒDEMATIS MALIGNI.*

B. œdematis maligni is characterized by a considerable stability in regard to the pathogenicity of individual strains even after years growth upon artificial media. Different strains may show some degree of variation in pathogenicity but the range of divergence in this respect is much less than that found in *B. welchii* or *B. novyi*. Pigeons, guinea-pigs, mice and rabbits, also dogs, are all susceptible to infection.

If 0·02 to 1 c.cm. of an actively growing culture, according to the virulence of the strain, is inoculated into the thigh of a guinea-pig, the animal dies in from 12 to 24 hours, with an extensive œdema and the formation of gas in the tissues. If the dose given is sublethal the animal seems unaffected by the inoculation. If, however, the organisms proliferate and an illness is set up, death follows invariably. There appear to be no cases of spontaneous recovery of guinea-pigs from an established infection with *B. œdematis maligni.*

Post-mortem appearances. On post-mortem examination an extensive blood-stained œdema of a deep colour is seen. A considerable amount of gas is developed in the areolar tissue round the muscles involved, which show a characteristic very deep red colour. As distinguished from the condition seen in an infection with *B. welchii* the muscles are neither friable nor soft, nor have they a sodden digested appearance. There is no putrid odour. There may be a collection of fluid in the peritoneal cavity and in the pericardium ; this is not as a rule very evident in guinea-pigs which have died in 12 to 24 hours, but is well marked in rabbits which have succumbed to the disease after 24 to 48 hours. The suprarenal glands may show some redness, but this is a variable feature and is never so marked as in the case of *B. welchii* infections. The suprarenal glands are more frequently affected in mice.

Microscopic examination. Microscopic examination of the exudate and more particularly of smears from the affected muscles show very numerous motile rods and usually also the characteristic ' navicular ' and ' citron ' types. Swollen club-shaped rods and short chains of intensely staining bulb-shaped individuals may be seen. Spore-bearing rods are not as a rule to be seen until some hours after death. The peritoneal surface of the liver shows long snake-like filaments, which are of diagnostic importance and are one of the points of distinction between this organism and *B. chauvœi.* In mixed infections of *B. œdematis maligni* and other spore-bearing anaerobes ' citron ' types in the muscles and the long filaments on the peritoneal surfaces are not always seen. The distribution of the organisms in the body is much the same as that already given for *B. welchii.* There is a very early invasion of the blood stream and the liver becomes in particular a focus for the multiplying organisms.

PATHOGENIC ACTION OF *B. NOVYI.*

B. novyi is a very toxic organism and is less of an invader by actual weight of numbers than either of the two species already described. The pathogenicity of different strains for laboratory animals varies somewhat and there is a certain tendency to loss of virulence, though strains isolated in 1918 were still pathogenic eight years later. Guinea-pigs, mice, rats and rabbits are all susceptible to infection with *B. novyi.*

The injection of 0·25 to 1 c.cm. of a 24-hour broth or glucose broth culture into the thigh muscles of a guinea-pig produces death within 24 to 48 hours. Examined immediately after death, the muscles at the site of inoculation are red and somewhat softened but not diffluent. As a rule very little, if any, gas is developed. There is a thick spreading gelatinous œdema, which is usually quite colourless, but if the infection has taken a very rapid course the œdema may be tinged with pink. Some strains are much more hæmolytic than others and extensive petechial hæmorrhages may be produced in some cases. The bacilli, among which pleomorphic forms may be seen, do not form spores in the body during life and are to be found in numbers only at the site of inoculation ; the œdema fluid and the peritoneal surfaces of the liver show only infrequent individuals. Cultures from the heart-blood are positive except in those cases where the animal has died from the effect of the toxin injected with the inoculum, and not from an actual infection with the bacilli.

PATHOGENIC ACTION OF *B. CHAUVŒI.*

B. chauvœi is pathogenic for guinea-pigs, mice and pigeons, but white rats and rabbits show considerable resistance. If 0·2 c.cm. to 1 c.cm. of a glucose broth culture, or the supernatant broth from a meat culture, or a glucose meat culture, are injected into the thigh of a guinea-pig, death ensues in 18 to 60 hours. The post-mortem appearances resemble those of *B. œdematis maligni*. A certain amount of blood-stained œdema is produced, and the muscles at the site of inoculation are of an intense deep red colour. There is, if anything, more hæmorrhagic staining of the tissues and the œdema and less gas than in infections with *B. œdematis maligni*, while there is less peritoneal exudate in the case of *B. chauvœi*. The heart-blood is invaded as a rule, unless the course of the disease has been very rapid. Long filaments are not formed upon the peritoneal surfaces of the liver. At the site of inoculation many rods and also bulbous and club-shaped forms are seen. Spores are formed after death but are not as a rule present during life. There is a greater elegance and slenderness in the shape of the 'navicular' types in *B. chauvœi* than in *B. œdematis maligni*.

PATHOGENIC ACTION OF *B. FALLAX.*

This organism loses its pathogenicity so rapidly upon isolation upon artificial media that its pathogenic action has not received much study. Weinberg and Séguin (1918) give a description of the illness caused in guinea-pigs. Henry and Lacey (1920) also report a gangrene in guinea-pigs inoculated with cultures from muscle fragments from cases of gas gangrene which was apparently due to *B. fallax*. As already stated, this organism can cause a fatal gangrene in man, and was isolated from the blood stream during life as the only organism in certain cases.

Three strains of *B. fallax* were examined by Weinberg and Séguin (1918) immediately after isolation, and were pathogenic for mice and guinea-pigs but not for rats ; a few weeks later they were no longer pathogenic. The illness produced by the injection of 1 c.cm. of broth culture ended in death in 16 to 24 hours. The post-mortem examination showed the muscles at the site of the inoculation to be red and injected, with a good deal of gas in the tissue ; there was a blood-stained œdema less firm in consistency than that found in an infection with *B. novyi*.

The organisms were fairly frequent both in the affected tissue at the site of inoculation and in the serous exudate. The bacilli were extremely motile. Spores are not formed even after death. The organisms were found on the peritoneal surfaces and in the peritoneal exudate; they were in isolated groups and short chains, but filaments were not as a rule seen. The general picture of the disease was that of a gas phlegmon. A rather feeble toxin was obtained from the cultures which produced death in guinea-pigs when inoculated intravenously; it was not hæmolytic.

PATHOGENIC ACTION OF *B. HISTOLYTICUS*.

The pathogenicity of the strains of *B. histolyticus* is apt to diminish with the passage of time upon artificial media, but Beckwith and MacKillop found one of Weinberg's original strains isolated some time before 1918, still actively pathogenic in 1923–4 in doses of 2 c.cm. Hall in 1923 isolated two strains from the intestine of man which were highly virulent; the virulence was as great in these strains of intestinal origin as in those derived from cases of gas gangrene. Torrey (1925) also isolated two strains from the ileo-cæcal region in man on two occasions.

The injection of an infecting dose (0·5 to 2 c.cm.) of a 24-hour glucose broth culture into the thigh of a guinea-pig produces an extensive digestion and disintegration of the living tissue. A swelling occurs at first at the site of inoculation, and a hæmorrhagic infiltration with small clots of blood spreads over part of the abdomen. The tissues inoculated begin to break down, the skin disintegrates, and in 18 to 24 hours the bone of the limb is denuded. It is remarkable that an organism so feebly proteolytic, judged by its action *in vitro*, should have so formidable a capacity for the digestion of living tissue. The animal dies within 48 hours as a rule, but in rabbits, after intravenous injection, death occurs in the course of 10 to 15 minutes (Weinberg and Séguin, 1918). A close study of the effect upon the tissues has been made, but this is dealt with later under the heading of the toxins; it is enough here to remark that the toxic effect is not restricted to the local lesion. The bacteria are found in considerable numbers in the different muscles and in the hæmorrhagic exudate. They are also found on the peritoneal surfaces in groups and in chains, but filaments are not formed. The organism does not form spores as a rule in the living body, but a few may sometimes be found in the older lesions if the animal survives for upwards of two days. There is no odour of putrefaction.

ORGANISMS CONCERNED IN GAS GANGRENE BUT NOT PATHOGENIC IN PURE CULTURE.

B. tertius may be dealt with first, not because of its greater importance, but because it is the only purely saccharolytic organism in this particular subdivision of the anaerobes. It has no actual pathogenic action so far as is known; it has, however, a very rapid growth and produces acid and gas under conditions in which only small quantities of sugar are available. It has some share in the production of local infections with the evolution of gas. It is very frequently present in great numbers in association with *B. welchii* and also with proteolytic organisms, and it is sometimes carried into the blood stream.

B. aerofœtidus has been considered by Weinberg and Séguin (1918) to be pathogenic when quite newly isolated, and a white gelatinous œdema followed by death was produced in guinea-pigs. There was no hæmolysis and the odour was putrefactive. This pathogenic action has not been confirmed by other workers.

B. bifermentans, and particularly *B. sporogenes*, under which for the present purpose are comprised the closely allied species of *B. parasporogenes* and *B. centrosporogenes*, have a quite special importance in all anaerobe diseases, and especially in gas gangrene in man and in the allied infections of animals. They undoubtedly play a definite role in producing the large number of putrid mixed gangrenes where the putrefying bacteria not only cause local damage but are carried into the blood stream.

It is stated by Weinberg and Séguin and by McIntosh (1917) that *B. sporogenes* is sometimes pathogenic in pure culture for guinea-pigs, though only in large doses. Such cultures have not come within the experience of the writer, but Weinberg returned to the study of this type (Weinberg and Howard, 1927) and confirmed his earlier findings of strains which are pathogenic on first being isolated.

The admixture of *B. sporogenes* with any of the pathogenic anaerobes increases the infectivity and virulence so that sublethal doses of the pathogenic organism becomes lethal. These mixed putrefying gangrenes can be produced experimentally and have a definite appearance, differing from those already described by the darker colour, the greater destruction of all the tissues, including the skin, and the putrefying odour. The mixed type of gangrene was well known during the war. The blood cultures from gangrene in the early months of the war contained very frequently, in the writer's experience, a mixture of *B. welchii* and *B. sporogenes;* sometimes even of *B. welchii*, *B. tertius* and *B. sporogenes*. Later on, probably owing to differences in surgical treatment, the putrid form of gangrene seemed to be less frequent.

Some light is thrown on these putrid gangrenes by Blanc and Pozerski (1920[1]); they showed that *B. sporogenes* could effect only a very slight degree of damage to normal healthy tissue, but if acid is injected into the muscle first its power of attack is very greatly enhanced. The pathogenic and some of the non-pathogenic organisms such as *B. tertius*, produce considerable quantities of acid in the presence of muscle sugar, and a symbiotic action between these organisms and *B. sporogenes* in the living body seems indicated.

SOLUBLE TOXINS.

The evidence for the existence of soluble toxins in the case of the pathogenic anaerobes is very convincing. The particular interest lies in the variation in the nature and type of toxin produced by the different members of the group. A toxin for the present purpose is defined as a soluble non-dialysable bacterial growth-product which is destroyed by heat, and which, when injected in repeated doses into animals, acts as an antigen, calling forth in their serum an immune body which neutralizes the action of the toxin and prevents infection with the living organism in question. The description of the pathogenic action in a susceptible host and the stipulation of a latent period are purposely omitted from the definition.

The toxins of the gas gangrene anaerobes were recognized in certain cases at a very early stage in the history of the research carried out upon them, but no real understanding of their nature and action was arrived at for two reasons. In the first place they did not tally in their behaviour with the two well-studied toxins of diphtheria and tetanus, and were, therefore, discarded as not being true toxins; and in the second place the fact that anaerobes do produce also poisonous substances which are not antigens and not toxins confused the results and checked the progress of experimental work.

An example of the distinction between these two types of substance is afforded by the work of Blanc and Pozerski (1920[2 & 3]). They worked with *B. sporogenes* and *B. histolyticus.* They demonstrated proteolytic ferments in filtrates from cultures of both these species and tested them *in vitro.* They further showed that in general character these bacterial ferments, though slower in action, had much the same proteolytic powers as the diastases pepsin, trypsin and papain. It is known that small quantities of normal horse serum inhibit tryptic and papainic digestion. The authors added 0·1 c.cm. of normal horse serum to the filtrates from *B. sporogenes* which had already been shown to digest gelatin with great activity. The addition of the serum entirely inhibited the digestion. Ten times the amount of horse serum used in the same way had no appreciable effect upon the digestion of gelatin by the *B. histolyticus* filtrate. On the other hand the injection of the *B. histolyticus* filtrate into a horse produced a serum which had the most powerful inhibitory effect upon the digestion of the test object by the filtrate derived from the same species. Thus the filtrates both contain active proteolysins but of different character. The filtrate of *B. sporogenes* does not act as an antigen, whereas that of *B. histolyticus* does. The first cannot be considered to be a bacterial toxin and the second can be so classed.

In all the pathogenic anaerobes of this group the fundamental action of the toxin seems to be an attack upon cell tissue which evokes no inflammatory response except that involved in the destruction of the cell.

Toxin of B. welchii.

The soluble toxin of *B. welchii* was first obtained in a clearly recognizable form by Bull and Pritchett (1917[1 & 2]). They found that the filtrate of an 18 to 24-hour culture of *B. welchii* grown in 0·2 per cent. glucose broth to which fresh sterile muscle tissue had been added, produced death in guinea-pigs in intramuscular doses of 0·3 to 3 c.cm. according to the virulence of the strain and the conditions of filtration. Later workers (see p. 286) obtained stronger toxins which were lethal upon intramuscular injection of such doses as 0·3 to 1 c.cm. for a mouse, 0·2 to 1 c.cm. for a pigeon, and 0·2 to 3 c.cm. for a guinea-pig. Intravenous injection of toxin into mice will cause death in doses of 0·01 to 0·1 c.cm.

Filtration is a serious difficulty in all this work on anaerobes, as the toxin is liable to be retained in the filter. (In the writer's experience Seitz filters are suitable.) Bull showed that the filtrate contained a true bacterial toxin which was thermolabile, being destroyed by heating to 70° C. for 30 minutes ; an antitoxin could be produced by injecting the toxin into suitable animals, and this antiserum would neutralize the toxin in multiple proportions. He already distinguished two elements in the toxin, a hæmolysin and what he described as the locally injurious constituent, later called the myotoxin by Henry. The antiserum neutralized both elements. Bull also found that he could protect susceptible animals up to the eleventh day by injecting immune serum prior to the inoculation of living culture. The animals withstood 20 lethal doses of living culture on the eleventh day, by the fourteenth and fifteenth day the protection seemed to have disappeared, though the experiment did not show if the animals were as susceptible as untreated individuals.

Nature of B. welchii *toxin; hæmolysin and cytolysin.*

The growth of *B. welchii* produces a composite true toxin and in addition in sugar-containing media a non-specific histamine-like substance, thermostable and having a physiological action. (See above, Kendall and Schmitt, 1926 ; Kojima, 1922, 1923[3].) The importance of this substance is not yet clear, but there is evidence of its presence in some of the products obtained from cases of disease (Wassermann and Ficker, 1922 ; Williams, 1926).

The toxin of *B. welchii* is somewhat difficult to produce, even from highly virulent strains. It can, however, be demonstrated in glucose broth, also in meat medium ; for methods suitable for its production in large quantities the reader is referred to p. 286. The reaction of the medium is always of great importance and the moment at which the fluid is filtered off is also significant (Bull and Pritchett, 1917[2]). The true toxin contains at least two elements, the hæmotoxin (hæmolysin) and the killing toxin (cytolysin) called the myotoxin by Henry. Both these substances have antigenic properties. *B. welchii* toxin can be dried by precipitation with ammonium sulphate followed by resolution and a secondary precipitation with alcohol (Henry and Lacey, 1920[1] ; Henry, 1922 ; Kadisch, 1923). Henry, in his experiments in 1923, came to the conclusion that the hæmolysin deteriorated by this process of drying to a greater extent than the myotoxin, while the storing of the filtrate in the cold gave the opposite result, i.e. the lethal factor, which he identifies with the myotoxin, deteriorated more rapidly than the hæmotoxin. He also reported that the myotoxin could be absorbed to a certain extent by placing it in contact with fresh muscle tissue; dried muscle and cooked horse muscle, also fresh liver and kidney of guinea-pigs produced no such change in the toxicity of the filtrate. The precipitated toxin was unaltered by contact with fresh muscle.

It is not possible in a description of the action of *B. welchii* toxin actually to treat the two elements in the toxin separately, their relation and interaction not being as yet sufficiently well understood. Some special study of the hæmolysin has, however, been made and the results are given below.

Wuth (1923) (see also Kojima, 1922) studied the hæmolysins of *B. welchii in vitro*; they appeared after 24 hours' growth in 2 per cent. glucose broth containing precipitated chalk, but further growth of the culture tended to destroy them. He carried out his experiments with bacteria-free filtrates. He shows the lysin to be thermolabile and very fragile, especially when exposed to air; a lysin that had lost its potency did not increase the friability of corpuscles subsequently exposed to an active *B. welchii* hæmolysin. Wuth found that the hæmolysin was readily absorbed by powdered fibrin, by erythrocytes, and by minced-up tissue from various organs. He obtained evidence that it was lipotropic: quite small quantities of lecithin very definitely lowered the hæmolytic capacity. The hæmolysin was present in the filtered exudates from infected guinea-pigs. It could be neutralized by anti-welch serum, whether derived from cultures or from exudate: a strictly specific phenomenon, for normal sera and sera derived by the injection of antigens from other anaerobic species have no inhibitory effect.

In all the tests with the hæmolysins of *B. welchii* the reaction of the fluid in which the cells are suspended is of the utmost importance, the haemolysin losing its potency if the solution is too alkaline. A pH of 7·0 is the optimum, and 7·5 to 8·0 too alkaline. This is a point to which Neill (1926) draws attention, showing that it is a specific quality of *B. welchii* hæmolysin; tetanolysin is much less susceptible to variations in the reaction. This worker makes the interesting suggestion that the reaction of normal blood is not the optimum for the hæmolytic action of *B. welchii* lysin and that this may afford some protection against *in vivo* hæmolysis. The effect of even a transitory or local acidosis may, therefore, be of considerable importance in the blood destruction.

Neill finds that *B. welchii* hæmolysin is inactivated by exposure to air and that this is a true oxidation phenomenon; moreover, it is reversible, and the lysin can be reactivated by the reducing action of bacteria or of sodium hydrosulphite. *B. welchii* hæmolysin is inactivated by exposure to 55° C. for 10 minutes; this is a shorter time than that recorded by other workers, but it appears that no actual experiments were carried out by Bull, Henry or Wuth to ascertain the lower time limit. The reaction did not seem to alter the time taken to destroy the lysin by heat. When inactivated by heat no reactivation could be effected by exposure to reducing agents. Neill further found that the oxidized (inactive) hæmolysin had the same heat resistance as the reduced (active) lysin. The oxidation process takes place more rapidly in a system of pH greater than 7·0. Reed, Orr and Campbell (1927) confirmed Neill's work as regards the loss of hæmolytic action in oxidized *B. welchii* toxin and added the observation that a much higher concentration of the toxin was required to hæmolyse oxygenated red-cell suspensions than reduced red-cell suspensions. This is not so with other hæmolytic agents.

Action of B. welchii *toxin.*

The pathogenic action of *B. welchii* in the body shows the combined effect of the two elements considered in the preceding paragraphs. It is again not possible in the present state of our knowledge to make a clear separation of the action of the hæmotoxin from that of the myotoxin. The word myotoxin used by Henry is convenient, but it should be clearly understood that the element so designated will attack the cells of other tissues.

Action of B. welchii *toxin in gas gangrene and histological changes in acute cases.* The action of the circulating toxin is partly hæmolytic and partly consists in an attack upon cellular tissue. The blood destruction in man can be observed in varying degree by the local bronzing of the skin and the peculiar ashy grey colour during life, also by methæmoglobin in the urine and dark staining of the serum, and by the thromboses in the blood vessels and the hæmosiderin in the spleen after death. Kettle remarks that the thrombi are to be found in the smaller vessels all through the body, and that granules of pigment may be present in quantities in the endothelial cells of the liver and in the renal epithelium. Nürnberger (1925) and Lehmann (1926) noted the effect of the great blood destruction on the kidney in cases of puerperal sepsis; the tubules were sometimes actually blocked with hæmoglobin cylinders.

In material from human cases, Kettle describes degeneration phenomena in the cells of the liver and kidney; occasionally there is fatty degeneration, but much more generally a cloudy swelling up and disorganization of the cell body.

The spleen shows changes due to the altered condition of the blood such as œdema, thrombosis and an occasional increase in the macrophages; in some instances the malpighian corpuscles are hypertrophied and exhibit a proliferation of endothelial cells. Kettle notes that this is a reaction to the toxæmia and is not due to local proliferation of bacteria, and recalls the similar condition appearing in cases of diphtheria.

In the acute death which takes place when rabbits receive a lethal dose of toxin injected intravenously the action on the heart muscle was considered to be the probable cause of death, but Kettle does not find any very marked alteration of heart muscle beyond a certain amount of fatty degeneration in the post-mortem material examined, except where local foci were present. Nevertheless, in cases of gas gangrene the actual cause of death seems very frequently to be a quite sudden heart failure. Lehmann (1926) notes the definite parenchymatous degeneration of the heart muscle in a fatal case of puerperal sepsis caused by a generalized *B. welchii* infection.

An important feature in the action of the toxin of *B. welchii* and also of *B. œdematis maligni* in acute infections is the exhaustion of the suprarenal glands (Bullock and Cramer, 1919). In mice there is a congestion of the cortex, and by Cramer's osmic vapour method there is shown to be a diminution of the cortical lipoids and a disappearance of the adreneline from the medulla. Similar changes are stated to occur in cases of gas gangrene in man.

There does not seem to be any histological evidence that the central nervous system is affected by the toxin except where there is an invasion of the meninges by the bacilli; this, however, can take place. At the same time the lipotropic character of the hæmotoxin should be considered in this connection. The view of Kettle that the central nervous system is not directly affected by the toxin in acute cases is supported by Fraenkel and Wohlwill (1922), who investigated a small series of deaths from *B. welchii* infection in a civil hospital. Anders (1917), however, claims that there are important degenerative changes in the ganglion axis cylinders, which he attributes to the action of the toxin. He finds, in agreement with Kettle, that there is little change in the heart muscle.

Action of the circulating toxin and changes produced by chronic infection without gangrene. Cornell, as has already been mentioned, produced a chronic infection in rabbits, and made an analysis of the action of the toxin. He found a chronic anæmia with anisocytosis and established that this is a specific effect. Inoculation of living culture into the spleen is followed by the appearance of anisocytosis in 6 to 48 hours, and the condition lasts till the death of the animal, as much, perhaps, as 5 months later; if washed bacilli are inoculated subcutaneously the blood change is first seen in about 3 weeks and continues till death. If *B. welchii* antitoxin is injected with the living culture into the spleen the early blood change does not take place, the effect is, however, produced 10 days to 2 weeks after the injection, and this is a specific phenomenon; normal horse serum and other antisera having no inhibitory effect upon the early blood changes. Cornell further shows that the alteration in the blood is apparently not due to the local effect of *B. welchii* upon the spleen, but to the direct action of the circulating toxin upon the blood cells. He demonstrated this by the following experiment: if both the jugular veins of a rabbit under urethane anæsthesia are tied off so as to make two living blood tubes, and toxin is injected into one and not into the other, smears from the former will show anisocytosis after 1 hour, while those from the uninjected vein will give a normal appearance. Cornell considered the anisocytosis to be a stage in the actual hæmolysis. The change in size of the blood corpuscles is further confirmed by measurement in the *in vitro* experiments of Orr and Campbell (1928) (unpublished communication). They consider it to be a specific action of *B. welchii* toxin, but their results suggest that elements in the toxin other than the actual hæmolysin are responsible for the characteristic change in the size of the cells. Cornell calls attention to the resemblance between the blood picture produced in this way and that found in pernicious anæmia, and recalls Herter's (1907) hypothesis connecting *B. welchii* with that disease.

Kahn and Torrey (1925) confirmed Cornell's work by injecting *B. welchii* toxin intravenously into monkeys in appropriate doses. They produced a marked anisocytosis with many macrocytes and a moderate degree of poikilocytosis, nucleated reds appeared as the injections continued, some with the morphology and staining reaction of megaloblasts, together with punctated basophils and polychromatophilia. At the period of greatest anæmia these animals showed some desquamation of the epithelium of the tongue and nervous irritability alternating with lethargy. The animals finally developed a solid immunity which could not be broken down.

Rabbits treated in this way show similar blood changes, but the immunity produced is transitory, lasting from 4 to 7 months (Reed and Spence, 1927). In Cornell's work, and in that of Kahn and Torrey, it should be noted that the actual killing toxin is never present in sufficient amount or sufficient concentration to produce its acute effect, but it is not clear that the results recorded in these chronic cases are due to the hæmotoxin alone.

Other effects of B. welchii *toxin.* Williams (1926) puts forward the idea that the toxæmia in cases of intestinal obstruction results at least in part from the absorption of toxin of *B. welchii* which is proliferating in the stagnant contents of the small intestine. Another instance of intoxication from the viscera is afforded by the infection of the gall bladder with this organism, which occurs occasionally in cholecystitis (Gilbert and Lippmann, 1902 ; Halle and Marquezy, 1922 ; Baugher, 1914 ; Kirchmayr, 1925 ; Brütt, 1923 ; Pearce Gould and Whitby, 1927). In addition to the effects already described, *B. welchii* toxin seems to have, according to the generally accepted view, a paralysing action upon phagocytosis, but no very accurate or detailed work seems to have been done upon this aspect of the toxin.

The existence of a true neurotoxin is implied by several workers, but it has not as yet been demonstrated ; the suggestion of some such action is considered to be implicit in the clinical picture, and Wuth's discovery of the action on lecithin would merit further investigation (Wuth, 1923).

Virulence and toxicity of B. welchii, *and variation.*

There is a very great variation in the virulence and toxicity of different strains of *B. welchii*, and of the same strain at different times. There appears to be a root strain quality which expresses itself in the capacity to form toxin. This seems to be a definite attribute, but conditions for its manifestation may not arise, and there is thus an apparent loss of toxicity, but in the writer's experience it can be demonstrated once more if the strain is properly treated. The capacity for rapid proliferation, which is one aspect of virulence, seems to be an extremely stable character in the vast majority of strains, which explains why, given the suitable inoculating dose, even strains of low toxicity *in vitro* may give rise to serious and fatal gangrenes.

Toxin of B. novyi.

The toxin of *B. novyi* was first recognized by Weinberg and Séguin in 1915. It can be obtained from glucose broth cultures after about 6 to 10 days' growth, or from glucose broth to which autoclaved meat or fresh muscle have been added. It is much more concentrated than that evolved by any of the other gas gangrene organisms. A toxin can be produced in large quantities which, when filtered through a Chamberland candle, will kill a 20 gm. mouse in 24 hours in a dose of 0·0001 c.cm. It is a fragile substance and is best preserved *in vacuo* at a low temperature in the dark. It is heat-labile, being destroyed by heating to 50° C. for 30 minutes.

The action of the toxin has been less well studied than that of *B. welchii.* Death is produced in laboratory animals when it is injected either intramuscularly or subcutaneously. A thick gelatinous œdema is found at the site of the injection, and there is little necrosis. There is a variable amount of hæmolysis, but this is never a very marked feature ; petechial hæmorrhages occur in the neighbourhood of the injection.

B. novyi is an acutely toxic organism and the part played by the actual proliferation of the bacillus is much less than in the case of *B. welchii.* Strains vary in their toxigenic capacity but nearly all will elaborate a toxin of very considerable potency.

Toxin of B. œdematis maligni.

The toxin of *B. œdematis maligni* was recognized as far back as 1887 and 1888 by Roux and Chamberland, who showed that it had antigenic properties. Very little further progress was made, however, in its study until the war. (Ficker, 1917 ; Weinberg and Séguin, 1918 ; Straub, 1919 ; Robertson and Dale, 1920.) The toxin can be obtained in various ways after 24 to 48 hours' growth in glucose broth or in glucose broth with autoclaved or fresh muscle. In the writer's own experience it can be made very conveniently by putting pieces of liver, excised under sterile conditions from a guinea-pig chloroformed 20 to 24 hours after inoculation with a strain of the organism, into flasks of digest broth or of 1 per cent. glucose broth. The moment at which the fluid should be removed from the open incubator and centrifuged as a preliminary to filtration is gauged by the auto-agglutination and precipitation of the bacteria. This takes place in about 22 hours if glucose broth is used and in about 44 to 45 hours in the digest broth without glucose. McEwen (1926) improved upon this by putting the liver into a parchment-diffusion shell and inoculating the broth. In making toxin the important factor is the reaction of the broth, which should be adjusted to a pH of 7·6 to 7·8. The broth should be autoclaved before the reaction is adjusted and the requisite pH obtained by adding sterile alkali before inoculation. In the writer's experience every strain of *B. œdematis maligni* will give an equally powerful toxin if the reaction is correctly adjusted, quite irrespective of the length of time which has elapsed since isolation. The filtration of the toxin presents some difficulty as the candle has a tendency to retain the active substance. A Seitz filter gives good results. The filtered toxin can be kept for considerable periods up to 7 or 8 months or longer if it is preserved in sealed vessels in the dark in a cold place. It is heat-labile and has antigenic properties.

The toxin is of low concentration and does not readily produce death upon intramuscular or subcutaneous inoculation ; it sets up a local œdema with a deep red staining of the tissue and a varying amount of local necrosis. Intravenous inoculation of doses from 0·1 to 2 c.cm. of toxin, according to its potency, into a 2,000 gm. rabbit kills the animal, with symptoms of respiratory disturbance, paralysis and convulsions, in from 3 to 15 minutes. When a smaller dose is used a delayed effect may be evoked and death occur in from 1 to 10 or 12 hours, but the delayed results are uncertain and difficult to control. Acute death is in bacteriological literature usually considered to indicate the action of poisons which are not true toxins, but in the case of *B. œdematis maligni* it is due to the actual toxin. The specific nature of the substance can be demonstrated by the addition of small quantities of an immune serum obtained by inoculating the filtrate into a sheep or horse ; normal sera of various animals do not inhibit the lethal action.

The action of the toxin upon the cells of the body has not been the object of minute histological examination. Kettle was of the opinion that *B. welchii*, *B. novyi* and *B. œdematis maligni* had all a very similar effect as regards the actual result presented to the eye in sectioned material, but it must be observed here that the actual toxins of these different organisms are quite sharply specific and distinct as toxins.

The toxic action of *B. œdematis maligni* filtrates has been analyzed with the help of the kymograph by Straub (1919) and by Dale and Robertson (1920). There is a general agreement in the results of these observers. There is an initial rise in the blood pressure, followed by a disturbance of the heart's action and a fall in pressure ; the heart recovers and the pressure rises steeply ; thereafter the heart gets once more into difficulties, the blood pressure drops and the animal dies. Dale is of the opinion that ' there are clear indications of a double action in the acute effect of the toxin, a pressor action, presumably due to vaso-constriction and a poisoning of the heart muscle. . . . The action on the heart is probably wholly in the direction of impairing its activity and the complex curve is due to the conflict between pressor vasomotor action and depressor cardiac action. The action on the heart is certainly direct; section of the vagi does not affect it. Both actions may be due to the same substance or to different constituents of the toxin; both are due to constituents of the true toxin, neutralizable by antitoxin and destroyed by boiling'. Straub (1919) considered that the action of the toxin resembles that of poisoning with digitalis. He found that the excised heart of a frog could be used to register the effect of the toxin.

The toxin of *B. œdematis maligni* has a hæmolytic action less pronounced than that of *B. welchii*. The potency of the hæmolysin bears no constant relation to the lethal factor in the toxin as tested intravenously in mice. Certain samples of toxin were found of equal titre in regard to their toxicity for mice while differing widely in their hæmolytic capacity as tested by *in vitro* hæmolysis of the red cells of rabbits. (Felix and Robertson, 1928, unpublished results.)

The Filtrate of B. chauvœi.

The filtrates of *B. chauvœi* cultures have very interesting properties, but their analysis is as yet incomplete and their properties are differently described by different workers. Some of the discrepancies are due to impure cultures and imperfect methods or to confusion in the analyses, and it is not necessary to attempt to harmonize them. Other discrepancies, however, are found in well-documented and carefully controlled work, but they are reconcilable and of interest.

Kojima ($1923^{1 \& 2}$) and Basset (1925) found that the filtrates were of low toxicity but still in some degree definitely toxic. Basset produced local lesions in guinea-pigs with subcutaneous doses of 3 to 5 to 10 c.cm. Kojima, after considerable difficulty, finally got a weak but definite toxin in broth containing 0·1 to 0·2 per cent. of glucose with pieces of coagulated egg-white or pieces of fresh guinea-pig muscle. It is important to note that the toxin was at its height between the eighteenth and thirty-sixth hour of growth. He used a membrane filter and observed that the toxin may easily be retained in the filter. The action of the toxin is of the same nature as that of *B. œdematis maligni*, but more feeble; it acts rapidly or after a delay on intravenous injection in mice and guinea-pigs and produces a local necrosis when given intramuscularly. From the very similar pathogenic action revealed in blackleg and malignant œdema it is to be expected that the toxins would show features in common.

Both Kojima and Basset find that the toxin is heat-labile. Kojima notes that it is destroyed at 52° C. after half an hour. His test is the toxic action in the living animal. Kojima distinguishes a hæmolysin and a lethal factor, which last seems to correspond in general terms to the locally injurious factor of Bull and the myotoxin of Henry in *B. welchii* toxin, and both these factors are destroyed at the same temperature; both can be dialysed, but only with difficulty, and they are destroyed by the action of pepsin and trypsin. According to Kojima the hæmolysin produced by *B. chauvœi* can be completely precipitated by a half-saturated solution of ammonium sulphate, while the lethal element remains to some extent in the supernatant fluid. The toxins and the immune body that they call forth are specific and they are distinct from those produced by *B. œdematis maligni*. Kojima also found that non-toxic filtrates obtained from late cultures of *B. chauvœi* which have lost all their toxic action, can be used to activate washed spores and are also antigens capable of immunizing susceptible animals against infection with virulent total cultures of *B. chauvœi*. He got no such immunizing action in the case of similar non-toxic filtrates of *B. œdematis maligni*.

It may be stated here that all the authors who have treated this subject with sufficient attention to the underlying bacteriology are agreed upon the general antigenic and immunizing capacity of the bacteria-free non-toxic filtrates of *B. chauvœi*, and upon their specific nature. These filtrates are now widely and successfully used in the practical immunization of cattle. (See Kitasato, 1889; Schöbl, 1910, 1912; Gräub and Zschokke, 1920; Uchimura, 1921; Basset, 1925; Allen and Bosworth, 1924; Scott, 1925; &c.) Viljoen and Scheuber (1926) investigated the non-toxic filtrates of *B. chauvœi* which they obtained from a continuous active growth of the organism of three weeks' duration induced by the neutralization and re-enforcement of the medium. The substance they dealt with is present also in filtered fluid from animals ill with blackleg. It is the natural and artificial ' aggressin ' first studied by Roux, and shown to have antigenic properties capable of immunizing animals against an infection with blackleg. These filtrates are non-toxic; they are capable of activating spores; they are heat-stable, resisting heating to 95° C. for 30 minutes. The immunizing capacity of the filtrates after exposure to heat was most carefully and prodigally tested out in sheep.

It appears that Kojima and Basset were working with the soluble bacterial antigens of filtered broth cultures, which also contained a bacterial toxin of a feeble type but corresponding in its nature with other actual toxins, and that Viljoen and Scheuber were immunizing their animals with the heat-stable filterable bacterial antigens in the absence of an actual toxin. It is obvious that, of course, these two types of filtrates have certain antigens in common. Both the toxin-containing filtrates of Kojima and Basset and the heat-stable non-toxic filtrates of Viljoen and Scheuber are able to immunize in the degree necessary to prevent infection. It is significant, indeed very important to remember, that in the absence of any good method of titration the nature of the immune response remains unanalysed. A bacteriolytic serum reaction in the immunized animal may possibly be quite as effective in checking invasion as an antitoxic blood reaction in the case of an organism of the nature of *B. chauvœi*.

In another communication, Kojima (1923^4) gives an account of what he considers to be a new organism belonging to the ' Rauschbrand group ', producing a highly potent toxin. From his description the bacillus is clearly *B. novyi* or a very closely related type, although the author, who had seen Weinberg's work, failed to recognize the organism. Kojima isolated the bacillus from a case of blackleg and obtained also a

'Rauschbrand type Foth', i.e. *B. chauvœi*, from the material. (For the occurrence of blackleg due to *B. novyi*, see McEwen, 1927.)

The Toxin of B. histolyticus.

The toxin of *B. histolyticus* was first obtained by Weinberg and Séguin (1917), who recognized its two main actions, namely, an immediate acutely lethal property when injected intravenously and a local necrotic effect when injected intramuscularly. They described several of the characters of the toxin. It is formed very early in broth cultures after 18 to 24 hours' growth and is quickly destroyed; it has a tendency to be retained in the filter; the bacteria-free filtrate has the capacity to liquefy gelatin. The toxic substance is not hæmolytic, and the blood corpuscles in the hæmorrhagic lesions it provokes are not destroyed. Weinberg and Séguin also noted that the filtrates had antigenic properties.

Interesting and detailed work has also been done more recently upon the action of the filtrates and cultures of *B. histolyticus* by various investigators, notably Nasta (1922), Blanc and Pozerski (1920[1 & 2]) and Beckwith and MacKillop (1924). Blanc and Pozerski confirmed the antigenic quality of the filtrates of *B. histolyticus* cultures and also their capacity for *in vitro* digestion (see above, p. 262). Nasta found that centrifuged toxin, which was not, however, proved to be bacteria-free, produced upon injection into an animal a breaking down of the substance of the muscle, then a lysis of the nucleus and finally a complete emptying of the fibre, the sarcolemma alone being left. The muscle fibre fills with blood, which coagulates. He found that only the muscle was affected, and considered the action selective. Peterson and Hall (1923) record that sterile filtrates produce hæmorrhages with coagulation of the extravasated blood in guinea-pigs. Beckwith and MacKillop (1924) made a detailed study of the results of infection with *B. histolyticus* upon the organs of the body, and concluded that the effects are by no means confined to the local muscular lesions. They noted that while striated muscle tissue is disrupted the selective action of the toxin is not limited to this type of structure. Epithelial cells are destroyed in many different organs, as also connective tissue. There is a generalized intoxication with attendant tissue destruction; the liver, spleen, kidney, lung, suprarenals and heart muscle all reveal profound degenerative changes and it is pointed out that these are produced from a localized infection and from a primarily local condition. It is to be noted that fibrin is developed, and that in spite of the great local destruction of tissue there is no serious hæmorrhage at the site of inoculation.

It seems important to draw attention to the far-reaching toxic effects of an organism which is not actually acutely pathogenic.

The Toxin of B. fallax.

The toxin of *B. fallax* has not received much study. The early loss of pathogenicity upon cultivation make this species an unsuitable object for investigation by ordinary laboratory methods. Weinberg and Séguin

(1918) described a feeble toxin which kills guinea-pigs when injected intravenously in doses of 1 to 2 c.cm., while the subcutaneous inoculation of 3 to 5 c.cm. produces a characteristic œdema. There are no signs of the presence of a hæmotoxin. Mutch and Mutch (1927) found *B. fallax* in a case of arthritis and isolated it from the knee joint. They were inclined to think that it plays a role in the intoxication of certain types of this disease and used a *B. fallax* vaccine apparently with some degree of success. (See Weinberg, Renard and Davesne, 1926, for *B. fallax* and *B. histolyticus* in appendicitis.)

PATHOLOGY IN MAN.

Most of the features of importance have already been presented in the foregoing divisions of this section, but the particular reference of some of the data to the diseases occurring in man may now be mentioned.

Fulminating gas gangrene has already been described above, and the nature of the processes involved has been discussed. This condition occurs in wounds and in injuries; the injuries are nearly always of a major kind, involving destruction of tissue, crushing, laceration or compound fractures. More important from the point of view of disease and of civil medicine are the infections with anaerobic bacilli that have to be considered in addition to the wound gangrenes. The difference between the actual gangrenes and the anaerobic infections is one which runs through all the pathological manifestations of these organisms, and will be clearly seen too in the natural diseases of animals (e.g. braxy and malignant œdema in sheep). The site of the infection and the relation to the body are, it must be emphasized, the factors which determine the morbid condition. Thus local anaerobic infections of lesser wounds and injuries are restricted to a local cell destruction. They are in their inception mild infections without gangrene or appreciable intoxication, and they may never proceed further. Into this category belong the less serious and transitory contaminative infections of operation wounds, where the infection arises from the intestine. Here too, belong the transitory puerperal infections and infections developing as a complication of urinary diseases, certain types of bronchiectasis, lung gangrenes and the less serious anaerobic abscesses.

Generalized infections of a fulminating character may evolve from any of the preceding types and lead to a rapidly fatal end. Into this group go all the rapid septicæmias, the serious puerperal cases, the fatal bowel contamination of wounds, the fatal metastatic gangrenes and those cases where virulent anaerobes are injected along with therapeutic solutions into the often already enfeebled patient. Here also should be placed those infections of wounds quite trifling in themselves, with apparently unimportant local symptoms, where the patient dies from a rapid intoxication with great blood destruction. A closely allied group is made up of cases where there is an internal anaerobic focus of uncertain origin, leading ultimately to a very serious and often fatal generalized toxæmia. Here may be placed those cases of appendicitis where there is a local infection of the organ, and consequent upon this the anaerobes are present in the peritoneum. This condition has attracted a good deal of attention, and the authors who describe it seem to assume that the site of the evolution of the toxin is in the peritoneum and do not consider the possibility of it having an intestinal origin.

Finally come the cases of toxæmia without cell invasion. Here should be placed any form of absorption of anaerobic toxins from the intestine, and among them the serious condition which supervenes upon acute intestinal obstruction. Here also seems to belong the type of case described by Fraser, where an anaerobic infection in a serous cavity produces death from the absorption of toxin. Fraser notes the tendency of these cases to evolve slowly; the centre of infection usually situated in the pleura. The symptoms of the toxæmia appear relatively late and often with extreme suddenness.

It is necessary to direct attention to the fact that anaerobes are capable of producing chronic as well as acute conditions, and it should be noted that their action, even when they are restricted to a local position, may be far-reaching and may involve degenerative changes in the blood and the tissues of all the organs of the body, including the bone marrow. Anaerobe infections are not restricted to any particular tissue nor to any particular site in the body.

It should, perhaps, be remarked that in the account of the pathogenic action and in the analysis of the toxic effects, *B. welchii* has received a greatly preponderating share of attention. This is in the first place due to the fact that *B. welchii* has received much more study, and secondly to the apparently greater frequency of its occurrence in man and, therefore, in the material observed. The writer would, however, suggest that the other pathogenic anaerobes should be borne in mind in considering the anaerobic infections in civil practice. The pathogenic anaerobes other than *B. welchii* produced about 30 per cent. of the fatal gangrene in certain groups investigated during the war. The figures given by different observers varied somewhat, and the methods used in determining the causal agent must have influenced the results. (For anaerobic infections in civil practice produced by species other than *B. welchii*, see Weinberg, Renard and Davesne, 1926; Mutch and Mutch, 1927; Meleny, Humphreys and Carp, 1927; Ukil, 1922; Sordelli, 1922; Barney and Heller, 1922. The cases were all authenticated by the culture of the organism.)

The following list gives a few references to recent literature dealing with cases in civil practice illustrative of the conditions summarized in the foregoing:

Gangrenes similar in type to war gangrenes arising from injury or accident or from accidental infection by means of therapeutic injection, and also metastatic gangrenes: Eichler, 1926; Gage, 1926; Cramp, 1912; Lande, 1926; Kemkes, 1923; Wanke, 1926; Knauer, 1924; Fraenkel and Wohlwill, 1922; Batzdorf, 1925; Dayton, 1925; Learmonth, 1924; Pilcher, 1925.

Anaerobic infections arising internally, other than in cases of appendicitis, such as cholecystitis, gas abscesses, &c.: Snyder, 1924; Berkow and Tolk, 1923; Kirchmayr, 1925; Baugher, 1914; Pearce Gould and Whitby, 1927; Brütt, 1923.

Anaerobic infections in appendicitis and allied conditions: Heyde, 1911; Michel, 1923; Jennings, 1923; Williams, 1926.

Cases of puerperal sepsis: Nürnberger, 1925; Lehmann, 1926; Simon, 1922; Theobald, 1924; Marx, 1921; &c.

NATURAL DISEASES OF ANIMALS.

The pathogenic anaerobes are the cause of a number of diseases occurring in animals. These diseases are caused by the anaerobes already dealt with, and with the exception of *B. chauvœi* the organisms are all pathogenic, both for man and animals. The nature of the diseases give examples of the different possibilities of attack possessed by the pathogenic anaerobes.

B. welchii *in animals.*

B. welchii has been found not infrequently accompanying both infections with *B. chauvœi* and with *B. œdematis maligni* (Zeissler, 1920 and 1923; Zeissler and Rassfeld, 1923). Karman and Seifried (1924) are of the opinion that *B. welchii* can attack both cattle and sheep as an independent infection causing a fatal gas œdema of the muscles in these animals, and, in addition, a braxy-like condition in the fourth stomach of sheep. Nöller and Seeleman (1924) consider that *B. welchii* is the cause of a disease in cattle known in Germany as the ' Duerener cattle disease ' from the district in which it first arose. Lamb dysentery (Dalling, 1926) has been held to be produced by *B. welchii*, but further study has shown that the organism is apparently a new one, belonging to the spore-bearing pathogenic anaerobes; its exact character and position are not yet clear.

B. œdematis maligni *in animals.*

This organism is responsible for a number of diseases in animals. In the first place it gives rise to conditions which may resemble blackleg so closely that no clinical differentiation is possible. It also gives rise to acute septicæmias with local œdema of a very severe character in some position usually involving a fore or hind quarter, which can be distinguished in the opinion of veterinary surgeons from blackleg at autopsy. The distinctions rest upon the colour of the muscle, the less acid odour, and the appearance of the liver and kidney. It is, however, now generally held that the differential diagnosis between blackleg and malignant œdema in cattle can only be accurately established by a bacteriological investigation (Zeissler, 1923; Wagner, 1925; &c.). *B. œdematis maligni* is thus accepted as the causal agent of a disease in cattle resembling blackleg and of a similar disease in sheep. It has also been shown to set up the condition known as braxy or ' Bradsot ' in sheep (Gaiger, 1922; Grosso, 1913; Jensen, 1915; Hamilton, 1901; Nielsen, 1888). In this disease the characteristic lesion is in the fourth stomach, where a dark purple red patch of hæmorrhagic infiltration is found; this condition may spread to the duodenum and intestine, and sometimes to the other three stomachs. There may be a diphtheroid membrane over the hæmorrhagic area in the stomach. Microscopically a superficial necrosis is present; the necrotic area often shows Gram-staining bacilli; these may also be found at the site of the diphtheritic membrane. The blood vessels of the mucous membrane are engorged. The kidney generally shows degeneration and cloudy swelling of the cells of the tubules, hæmorrhages and some necrosis.

Köves (1918) found a very similar condition in swine caused by *B. œdematis maligni*. Here the disease was often restricted to the stomach, where a vivid red hæmorrhagic infiltration of the wall was evident. There was a certain amount of necrosis and a surface diphtheritic film usually containing many Gram-staining rods, which penetrated to a greater or less degree into the tissue of the stomach wall. Another manifestation of the same disease involved the muscles, generally of the foreleg. The condition ended as a rule fatally and very rapidly.

Meyer (1915) describes an epidemic of ' symptomatic anthrax ' in hogs in America, due to *B. œdematis maligni*.

Infections with *B. œdematis maligni* may arise in animals by way of the genital tract, often after abortion or after the birth of the young; the condition produced culminates in a general septicæmia. Young animals may be infected with the organism through the umbilical cord. Acute septicæmias can arise in various ways in most animals from injury or wounding, an interesting instance being the septicæmia of whales set up by means of the infected harpoon (Christiansen, 1920). Zeissler and Rassfeld (1923) are of the opinion that the ' harpoon poison ' consisted of a mixture of *B. œdematis maligni*, *B. chauvœi* and *B. sporogenes*. The symptoms were those of a gas gangrene of enormous extent.

B. novyi *in animals.*

Conditions of acute septicæmia can also arise in animals from natural infections with *B. novyi*. There are instances recorded in horses (Heller, 1920) and also in pigs (Mejlbo, 1925). Infection with this organism is perhaps more frequent than is recognized at present. The condition would be classed along with those arising from either *B. œdematis maligni* or *B. chauvœi*. Kojima (1923) seems to have had this organism when he described the highly toxic bacillus from a case of blackleg; it was associated with *B. chauvœi* in a mixed infection. (Also McEwen, 1927.)

A disease of sheep known as ' Australian black disease ' has recently been investigated by Albiston (1927). (See also Gilruth, 1911, and Dodd, 1921.) It is a braxy-like condition which causes death with considerable rapidity, and without anything very obvious in the way of symptoms. The post-mortem conditions are those of a hepatic necrosis. The subcutaneous vessels may be injected and there is an excess of peritoneal fluid, either clear or slightly blood-stained. The intestinal vessels are injected and the mucosa of the abomasum is slightly reddened, but there is no sign of ulceration. The pericardial sac is distended with a clear fluid which may coagulate on exposure to the air; the lungs and kidney are congested. These animals often show infection with liver fluke, but not invariably. The author speculates as to whether the fluke play any part in the inauguration of the infection. An organism has been isolated in pure culture in a series of cases from the liver. It is very difficult to obtain growth on first isolating it. This organism is highly pathogenic for laboratory animals and is a very strict anaerobe. It is perfectly clear from the author's description that he is dealing with *B. novyi* of a type differing a little in fermentative capacity from the wound gangrene strains and somewhat more difficult to cultivate. The picture in the experimental animals is the characteristic one already described for *B. novyi.*

Turner and Davesne (1927), working in Weinberg's laboratory, carried on further investigations with Albiston's strains and showed that serologically the sheep bacillus is identical with *B. novyi* (*B. œdematiens*) in spite of the slight variation in the fermentations. The very serious difficulty in acclimatizing the organism to growth upon artificial media is confirmed by Turner and Davesne and is a point of some importance in considering the obstacles encountered in this type of research.

Blackleg.

A very great deal has been written upon the disease known as blackleg. So large a literature would hardly seem necessary were it not for the lively controversy that arose over the confusion between blackleg proper and malignant œdema. This has now been settled, and the position of *B. chauvœi* and *B. œdematis maligni* has been made clear; these two organisms are separate bacterial species, both of them can infect animals and can give rise to the clinical disease described as blackleg or 'Rauschbrand'. The clinical disease of blackleg can also be set up by *B. novyi* (McEwen, 1927).

True blackleg (Rauschbrand of Foth) is due to *B. chauvœi.* It is usually stated that the mode of entry of the bacillus is by means of a slight wound or injury, but evidence of other methods of infection have recently been brought forward; and while blackleg is usually characterized by a serious muscle lesion, this is not by any means invariably the case. Acute septicæmias without muscle lesions are cited by Scott (1925) and by Wagener (1925). Scott also provides evidence of infection by means of the alimentary canal, and quotes Ronca (1920), who produced infection with *B. chauvœi* in guinea-pigs by this avenue. The general type of the disease is a bacterial invasion with a focus or separate foci in the muscles of the fore or hind quarter or of the neck, but the focus may be situated internally or in the muscles of the heart. In some cases, moreover, the disease may arise as an acute septicæmia with an inflammatory endocarditis without any obvious involvement of the musculature. The characteristic condition of blackleg is produced by the accumulation of œdema fluid, and the alteration of the tissue due to the proliferation of the bacteria. Gas is evolved, but, as always with the anaerobes, it is a variable feature in individual infections. The muscles affected have a well-known dark purple-red or purple-black colour, and, in spite of the œdema, the muscles themselves may be of a rather dry consistency. In cases where there is not much gas the muscles are described as being spongy and sodden. There is often a dark red or blackish-red effusion in the body cavity. The kidney is generally reddened and shows hæmorrhages, and the liver seen immediately after death is slightly enlarged, brownish and full of blood; later on it shows greyish yellow foci. These are due to the post-mortem multiplication of the organisms in the liver tissue. (Kitt, 1887, 1903 and 1911 ; Foth, 1909, 1910 and 1911 ; Goss, Barbarin and Haines, 1921 ; Grosso, 1913 ; Markoff, 1911 ; Wagener, 1925 ; Francke and Goerttler, 1926, should all be consulted for accounts of blackleg.)

Blackleg is primarily a disease of cattle but it does also occur in sheep (Zeissler, 1922). Wagener (1925) notes a curious epidemiological paradox in the distribution of the disease in sheep and cattle. The sheep cases occur in districts which are free from blackleg in cattle, and not in those where cattle infection is common.

Blackleg does not seem to occur as a natural infection in pigs, the cases reported appear to be due to *B. œdematis maligni.* There is a conflict of opinion and much confusion as to whether horses do or do not become infected with *B. chauvœi* (Foth, 1910 ; Wulff, 1912) ; they are extremely susceptible to infection with *B. œdematis maligni*, and Wagener (1925), reviewing the evidence concludes that true blackleg does not occur in horses. (See also Heller, 1920.)

Resistance.

NATURAL SPECIES RESISTANCE.

Certain species seem naturally resistant or immune to infection with individual anaerobes. In some cases the resistance is only relative and can be broken down, in others it appears to be so strong that the animal can be described as definitely insusceptible to infection. There is no correctly recorded case of *B. chauvœi* ever occurring in man : the horse also appears to be immune, the few recorded cases do not stand a searching criticism and are probably due to *B. œdematis maligni.* The rabbit is very resistant to *B. chauvœi*, but can eventually be infected with this organism. Cats are likewise insusceptible, and are very resistant also to infections with *B. œdematis maligni.* In most of these animals there is no information as to the nature of the immunity, whether they are unaffected by the toxin, or in what way they defend themselves. In the cat, however, some evidence is available. When the writer was studying the toxin of *B. œdematis maligni*, Dr. H. H. Dale made a test of the reaction in a cat under ether anæsthesia with the kymograph apparatus arranged to record the blood pressure. The toxin was injected intravenously and the animal received 12 c.cm. without any reaction whatsoever, except a faint response to the volume of fluid introduced. The same sample of toxin on the same occasion produced acute death in a 2,000 gm. rabbit in a dose of 2 c.cm. It is thus clear that the cat is simply not affected by the toxin under these conditions, even when it is introduced in the manner in which it is most easily absorbed by other animals. The nature of the defence remains unexplained.

Chauveau and Arloing (1884) found that the frog is insusceptible to infection with 'the organism of gangrenous septicæmia' provided the water it is in is kept at a temperature of less than 22° C. If the temperature is raised above 22°, the frog can be fatally infected by infection with this organism.

NATURAL RESISTANCE OF SUSCEPTIBLE ANIMALS.

There is a good level of resistance in all healthy animals to infection with the anaerobes, and this may be found coexisting in some cases with a rapid and invariably fatal issue once infection has been set up. Blackleg may be cited as an instance of this, and another example is the reaction of the guinea-pig to infection with the living culture of *B. œdematis maligni*; a sublethal dose produces no appreciable reaction, while an infecting dose is always fatal.

The pathogenic anaerobes of this group show a very poor capacity for proliferation in the body without some degree of local necrosis and tissue destruction, or of a pabulum of some kind, as, for instance, is found in the alimentary tract. Even when proliferation is taking place, the healthy tissues (McNee and Shaw Dunn, 1917), the sheath of the muscle fibres, also the endothelium of healthy blood vessels (as was shown by Arloing, Cornevin and Thomas, 1881 and 1887, for *B. chauvœi*) and undamaged mucous membranes are all valuable and on the whole efficient barriers to the spread of infection. When the production of toxin reaches even a very moderate degree of intensity the phagocytic action seems to be paralysed, but there is evidence of some attempt at phagocytic activity at certain stages of even fatal infections. Kettle (1919) noted the absence of inflammatory reaction, but also found that there was sometimes a leucocytosis in the subcutaneous and connective tissues; the leucocytes were of the polymorphic variety and actively phagocytic (see p. 256).

THE CAUSES OF THE BREAKDOWN OF RESISTANCE.

Practically speaking the breakdown is always in the first place due to the immediate effect of the absorption of the products of metabolism of the bacteria proliferating in some focus within the body. The relatively harmless saprophytic infections where absorption of toxin in significant amounts is not taking place are abundant testimony to this. The production of toxin even in small amounts is followed by serious damage to the cells at the site of proliferation. Once a devitalized area is produced by the anaerobe itself or by a co-existing aerobic infection, by failure of the blood supply or some other circumstance, the cytolytic action spreads and the invasion of living tissue may become formidable; and, while sound membranes, &c., may resist the action for a time, the balance in the end may be turned against them. The existence of a suitably alkaline reaction is probably a powerful factor in the accumulation of toxin. Once the toxin is circulating and setting up the widespread destructive action already described in parts of the body far removed from the original focus, the organisms which are carried by the blood stream can set up secondary foci, and the disease may evolve with great rapidity. The resistance against the toxin may be stubborn and only its accumulation and rise in potency or the attack upon certain vital processes (e.g. direct action on the heart muscle in the case of *B. œdematis maligni*) will bring about the catastrophic result. If intoxication is an early and serious feature, the body seems to put up no defence whatsoever.

Examples of all these conditions can be found in the descriptions given on pp. 252 et seq. In addition, the state shown in the chronic *B. welchii* infections described by Cornell (1925[1 & 2]) should be recalled as an example of a long drawn out but unsuccessful resistance to an organism which under other conditions creates a fulminating disturbance leading to a rapidly fatal close.

The Production of Immunity in Experimental Animals.

ACTIVE IMMUNITY.

In many types of bacterial disease an active immunity can be evoked in a susceptible animal by the inoculation of killed or attenuated bacteria. In dealing with the pathogenic anaerobes the use of heated and washed bacteria seems to be of limited application. In the case of *B. welchii* the writer (Robertson, 1916[2]) carried out experiments of this nature with guinea-pigs without finding any appreciable increase in their resistance to a lethal dose of the living culture. Even an already survived infection did not produce immunity against a subsequent inoculation. Hall (1922) is of the opinion that some degree of active immunity can be so produced by vaccination, but the account of his work leaves doubtful the solidity of the immunity. He has, moreover, taken too little into consideration the normal spontaneous recovery rate of these animals from *B. welchii* infections. Hall confirms Robertson's observation that recovery from an infection with this organism does not confer immunity upon a guinea-pig. The transitory nature of the immunity conferred upon rabbits by the injection of toxin of *B. welchii* in respect to the changes in the blood corpuscles may be recalled in this connection.

Immunization by means of formolized total cultures has begun of late to receive some attention (Weinberg and Prévot, 1925; Weinberg and Barotte, 1926; Leclainche and Vallée, 1925; McEwen, 1926; Karmann, 1927). These vaccines are prepared in various ways, but they contain the total culture, that is both the bacterial bodies and the soluble growth products. They would seem to be theoretically the ideal method of immunization in that they contain antigens of all the different classes evolved by the organisms. This work is, however, still in a very early stage of development and so far no very close or detailed analyses of the resulting immune response have been published.

Weinberg and Prévot (1925) prepared a series of vaccines with the pathogenic anaerobes by the following process. Cultures of *B. welchii* and *B. œdematis maligni*, grown for the production of toxin, are centrifuged until the supernatant fluid is quite clear; this is drawn off and treated with formalin to the concentration of 3 parts per 1,000, and incubated at 37° C. for 48 hours. At this stage it is an anatoxin comparable with the similar preparations made from diphtheria toxin. (See Volume VI for anatoxins in general.) The bacterial deposit is taken up in saline solution, formalin is added to a concentration of 5 per 1,000 and the solution incubated for 8 days at 37° C. The bacterial emulsion, thus sterilized, is combined with the anatoxin and the final mixture is used to immunize horses or the laboratory animals. The method is slightly varied in the case of the different species. Thus the bacteria and spores of *B. histolyticus* require to be left for 15 days at 37° in 5 per 1,000 formalin or in 10 per 1,000 for 8 days. *B. sporogenes* requires to be left in 3 per 100 formalin for 8 days, or in 20 to 25 per 1,000 for 17 days. With *B. novyi* (*B. œdematiens*), however, autolysis occurs so rapidly that the addition of formalin to a concentration of 3 per 1,000 may be made to the total culture and incubation carried out for 8 days; that is to say, that for this organism the total culture may be treated as for the preparation of the anatoxin of this species (Weinberg and Prévot, 1924).

The immune response to these formolized total cultures appears to be very good in the larger animals, while the reaction and inconvenience to the animal is greatly reduced (Weinberg and Prévot, 1925; Weinberg and Barotte, 1926).

In the case of *B. chauvœi* a dried blackleg vaccine was used extensively for many years and practical success was claimed for it, but the evidence was never very convincing, and the method has now been superseded. Leclainche and Vallée (1925), Karmann (1927) and McEwen (1926) advocate the use of a formolized total vaccine of *B. chauvœi* grown in broth as a means of immunizing against blackleg, and the result seems to be satisfactory.

Active immunity can be produced very successfully in animals by the use of bacterial filtrates; this was first done by Roux in 1888. He showed that the filtrates not only of bacterial cultures but also of the œdema fluid of animals infected with *B. chauvœi* and *B. œdematis maligni* (vibrion septique) had valuable antigenic properties, and that guinea-pigs could be effectively immunized with these materials. This was also shown by Schöbl in 1910 and 1912. Allen and Bosworth (1924) give an account of the immunization of guinea-pigs with toxin-antitoxin mixtures of

B. chauvœi either alone or combined with *B. œdematis maligni* so as to immunize at once against both these organisms. The injection of bacteria-free filtrates to immunize against *B. chauvœi* is widely used with great success (Uchimura, 1921; Kojima, 1923; Gräub and Zschokke, 1920; Nitta, 1918; Eichhorn, 1918; Basset, 1925; Viljoen and Scheuber, 1926; Haslam and Lumb, 1919).

It should be noted that if animals are immunized by these soluble bacterial products of *B. chauvœi* and *B. œdematis maligni* without the admixture of antitoxin, it is important that the filtrates should be sterile, otherwise the risk of infecting the animal is very great. The earlier practise of using whole cultures whose virulence was lowered by leaving them to stand for a number of days after removal from the incubator either failed to immunize because the toxin had deteriorated or were apt to cause infection because the living spores became active in the presence of the toxin. This method was first used by Kitasato (1889). With *B. chauvœi* the use of antitoxin seems less necessary, and is in many places omitted, as the bacteria-free filtrates have little or no deleterious effects. With *B. œdematis maligni* it seems advisable to use the toxin-antitoxin mixture, as, even apart from the risk of infection due to imperfect filtration, the bacteria-free filtrates themselves exercise a local action, producing slight swelling, reddening of the tissue, and some degree of pain.

The immune response in the case of the pathogenic anaerobes seems to show, wherever an analysis has been made, that the several elements of the toxin, such as the cytolysins and the hæmolysins, have antigenic properties. Wuth's work (1923) and Henry (1922 and 1923) have already been cited in the case of *B. welchii*, and Blanc and Pozerski (1920) for *B. histolyticus* (p. 272). This last is particularly interesting as the antiserum to *B. histolyticus* contains an antiferment.

The production of high titre sera by the injection of toxin is treated below, as also the practical application of various methods of immunization.

Passive Immunity in Experimental Animals.

The sera produced by the injection of the toxins of the gas gangrene anaerobes into appropriate animals have a high protective power. The titre of these depends upon the efficient immunization of the animal: under suitable conditions very active sera of high titre can be produced. The antisera against *B. welchii*, *B. œdematis maligni* and *B. novyi* have all been shown to confer a protective passive immunity when injected into laboratory animals against a subsequent inoculation with several times the amount of living culture required to produce death in the type of animal in question. In these experiments it is found that 2 to 3 c.cm. of a potent serum will prevent infection up to 10 days. It is a very striking experience to observe the entire lack of symptoms, often even of the faintest appreciable local swelling or tenderness in the protected animals, while the controls show the characteristic fatal lesions—the 'désordres épouventables' so well named by Pasteur. The sera are quite strictly specific, and working with pure sera no protection whatsoever is afforded against any but the same bacterial species as that by means of which the immune serum has been evoked. In practical diagnosis as well as in various kinds of experimental work these pure sera are of the utmost service. They can incidentally be used to sift out the active pathogenic agents in wound exudate, &c. (Henry and Lacey, 1920[3]).

The duration of passive immunity is variable and no really detailed experimental work seems to have been done, but a single dose of 2 to 3 c.cm. will generally protect a guinea-pig for 10 to 12 days and sometimes longer.

An immunity is transferred to the young of immunized animals. This was recognized in the case of blackleg by Kitasato in 1889. It appears to be in the nature of an active immunity, but is of a somewhat transitory character. Immunity is also conferred on the young animal by means of the milk of the immune mother; this is a passive immunity. A test was made by Koegel (1920) of the milk of a goat which had been immunized against *B. chauvœi*. He found that intraperitoneal injection of 3 c.cm. of the milk protected three guinea-pigs from a lethal dose of a highly virulent culture inoculated on the third day after the injection of the milk. The control animal died in 12 hours.

PRODUCTION OF SERUM ANTIBODIES.

The production of antitoxin has already been remarked upon, and the details of the process are dealt with below (see p. 285).

Precipitins.

At the present time the most contradictory views are held as to the value of the precipitin reaction in the study of the anaerobes. In the opinion of the writer investigation in this direction has been retarded by the confusion caused by the use of impure antigens and impure sera. A sound bacteriological basis is the necessary foundation of all work of this kind, and it would seem worth the while of workers to reconsider this aspect of the response to the anaerobes with a clearer outlook and a better technique.

Hecht (1913), in the case of *B. chauvœi*, uses immune sera and heated organ extracts. The test is made by placing the extracts derived from the muscle and organs of an infected animal or from cultures in contact with a specific antiserum derived from an immunized animal. A cloudy ring is formed at the junction of the serum with the extract. Gerlach (1921) criticizes the specificity of the reaction. This is also the criticism suggested by Gaehtgens's (1918) long and important contribution to the subject. Gaehtgens worked with wound strains which he divided into a 'Fraenkel-bacillus' group and an 'Œdembacillus' group. He found the reaction insufficiently specific, but his work suggests that the method might be of value if used with a clearer conception of the factors involved. Wagener (1925), from the point of view of its use in the study of blackleg, sums up against the value of the reaction, so also does Todorovitsch (1923).

Koegel (1920) found precipitins against blackleg in the blood of several untreated animals (sheep, goat and cattle), and studied their appearance in immunized individuals. He used heated organ extracts and considered the reaction to be specific. McEwen (1926) found the precipitin test applicable in the case of blackleg (*B. chauvœi*) and braxy (*B. œdematis maligni*). He made an immune serum in rabbits by the intravenous injection of saline emulsions of surface growths of *B. œdematis maligni* on Dorset's egg medium. His test consists in placing 0·2 c.cm. of saline, 0·2 c.cm. of a centrifuged (but unfiltered) toxin and 0·2 c.cm. of the rabbit immune serum in contact in a tube. The tube is rotated and incubated for 5 hours at 37° C. in the water bath, and the reading is made after an overnight sojourn in the ice chest. The author finds that this test distinguishes *B. œdematis maligni* from *B. chauvœi.* Carried out in this way, the test does not reveal the serological grouping of *B. œdematis maligni,* although the agglutination reaction does.

Weinberg, Prévot and Goy (1924) observed a flocculation of filtered toxin with (1) antitoxic sera, (2) with antitoxic and antibacterial sera, and (3) with a serum prepared from washed bacilli (the washing was apparently done with saline and without heating). The reaction seems to be specific, but there was no correspondence between the titre of the sera and the degree of flocculation. The experiments were carried out with *B. welchii, B. œdematis maligni, B. histolyticus, B. sporogenes, B. putrificus* and *B. bifermentans.*

Agglutinins.

Agglutinins can be called forth in the blood of experimental animals, particularly rabbits, by the intravenous injection of washed bacilli of many anaerobe species. Two important exceptions to this general statement are *B. welchii* and *B. tertius.* In the case of *B. welchii* auto-agglutination is a frequent phenomenon, but no good evidence of the production of agglutinins in the serum of the animals injected has been brought forward. McIntosh (1917) obtained strictly specific agglutinins to a number of species.

Certain species show serological grouping. *B. œdematis maligni* (Robertson, 1919–20) falls into at least three, and apparently four, agglutination groups. These groups were tested to see if the soluble toxins showed any similar division, but they did not : monotypical antitoxic sera produced from single strains neutralize the toxin from cultures belonging to each of the groups. These agglutination groups were distinguished by the results obtained from the large flaking agglutinins produced by the heat labile antigen. Small flaking agglutinins produced by the injection of heated bacillary antigen have also been demonstrated for *B. œdematis maligni* (Felix and Robertson, 1928). The small flaking agglutinins (stabilotropic) show a high degree of overlapping between the serological groups defined by the large flaking (labilotropic) agglutinins.

In the case of *B. novyi* the agglutinins appear to be restricted to the homologous culture, but no very extensive investigation has been made with this species. In so far as they have been studied the agglutinins of *B. sporogenes* also show a distinct division of the different strains into groups (Hall and Stark, 1923). N. and J. Mutch (1927) note the appearance of agglutinins for the homologous strain in the case of patients suffering from rheumatoid arthritis who have received injections of *B. fallax* vaccine.

Complement Deviation.

This method has been applied to the study of the anaerobes, but not very much work has been done. McIntosh and Fildes (1917) demonstrated complement-binding substances in the serum of animals immunized with *B. welchii.* Gaehtgens (1918) also used the method, but with doubtful success, due at least in part to insufficient care in obtaining pure bacterial antigens. Felix and Robertson (1928) investigated the complement fixation in *B. œdematis maligni* in relation to the large flaking and small flaking agglutinins. They found this method perfectly applicable, and their results showed that the complement-binding immune body is purely stabilotropic in nature, and that there is a complete correspondence between complement fixation and small flaking agglutination. The large flaking agglutinins are not in any way involved in the complement-binding reaction.

As in the case of the precipitin reaction, complement fixation is probably of wider application in the study of the anaerobes than would be supposed from the small amount of attention it has received. Both complement deviation and the precipitin reaction suggest themselves as of possible value in the study of the chronic forms of anaerobe infection, which are the subject of some recent investigations.

The Preparation and Testing of Toxins and Antitoxins: and Prophylactic Mixtures.

By R. A. O'Brien

(Wellcome Research Laboratories).

During the European War, in response to the military demand, much attention was given to the serological aspects of anaerobic pathogenic organisms, and large amounts of sera were prepared. During the period after the war little further interest was taken by those concerned with human medicine since the diseases caused by this group of organisms were thought to be rare in civil practice, and attention shifted mainly to the investigation of veterinary diseases, blackleg, braxy, lamb dysentery, &c. Recently, the possible importance of some of these organisms in various human 'civil' diseases, such as gas gangrene, abdominal conditions, abortion and pernicious anæmia, has again interested the serologist.

Certain general considerations apply to the production of toxins and antitoxins and prophylactic deratives or mixtures on the large scale, and to the testing and titration of these various substances, but as these are similar to those applying to diphtheria they need not be discussed here.

B. WELCHII.

Toxin.

Strain. It would appear that all strains of *B. welchii* obtained from different parts of the world make the same toxin and that one antitoxin will neutralize any *B. welchii* toxin. Strains vary in their readiness to produce toxin in ordinary media, and serologists responsible for the making of toxin on a large scale may at times find it very difficult to induce the strain or strains they are working with to produce good toxin with regularity. Repeated passage through guinea-pigs, pigeons or mice may raise the toxin-producing power of a strain. For the inoculum, some workers infect a pigeon or other susceptible animal intramuscularly with culture so that death occurs next morning; the excised injected muscle or liver is then inoculated directly into a suitable nutrient broth.

Medium. Toxin on the large scale has been made by the inoculation of young, rapidly growing cultures into nutrient broth. Ordinary peptone nutrient or tryptic digest broth (Hartley, 1922) with meat to a depth of about one inch at the bottom of the container, and adjusted to an initial pH of 7·8, is usually employed. It is not advisable to add glucose. Weinberg and Ginsbourg (1927, p. 170) use 'Vf' (peptic digest of liver with beef) and find that the resultant toxin kills a rabbit at an intravenous dose of 0·25 c.cm. to 0·5 c.cm. The medium, after autoclaving, can be kept in tall bottles until required for use. No surface seal is required. The growth is allowed to go on at 37° C. for some 18 to 24 hours, at which time the pH may be about 5 and toxin of reasonable titre may be produced. During filtration much toxin may be kept back or retained by the filter. If destined for the injection of horses, the toxin after filtration should be thoroughly tested for sterility. 100 c.cm. or more must be used in the test, for apparently a very small number of living *B. welchii*, when injected with large quantities of toxin into horses under immunization, may cause active gas gangrene.

Toxin is relatively unstable; it usually diminishes rapidly in toxicity at room temperature. Heating to 70° C. for one hour usually destroys all toxicity. Oxygenation causes rapid destruction (Lautenschläger, 1920; Buttle and Trevan, 1928). From the point of view of immunization there is no proof that this non-toxic toxin or 'toxoid' is not as good an antigen as fresh toxin. The freshly prepared toxic filtrate may be precipitated by 70 per cent. saturation with ammonium sulphate, dried and ground and sealed up; the powder apparently retains its toxicity and combining power unchanged for long periods.

Testing of toxin. Guinea-pigs, mice, rabbits and birds have been mostly used, the injections being made subcutaneously, intramuscularly, intravenously or intradermally (Klose, 1916; Glenny and Allen, 1921; Dalling, Glenny, Mason and O'Brien, 1928). Since the intravenous and intradermic methods allow of determinations over-night, and with both small degrees of difference of toxicity are detectable, these methods are much used in serology. A freshly made toxin, when injected intravenously into mice of 20 gm. weight, may kill in 12 hours at a dose of 0·01 to 0·1 c.cm. Of a good precipitated toxin 0·02 mgm. given intravenously will kill over-night. Large doses of toxin, e.g. 0·5 c.cm., kill within 10 to 15 minutes. The technique of the intradermal method is the same as that used in Römer's method for the detection of small doses of diphtheria toxin; the lesion is necrotic, appears in 24 hours and gradually heals. The m.l.d. by the intramuscular method, quoted by various workers, range from 0·3 to 1·0 c.cm. for the mouse, 0·2 to 1·0 c.cm. for the pigeon, 0·2 to 3·0 c.cm. for the guinea-pig.

Many workers (Bull, 1917; Weinberg and Nasta, 1920; Henry, 1922 and 1923) have shown that *B. welchii* secretes in broth a hæmolysin which lyses the corpuscles of the sheep, mouse, &c. It is readily destroyed by heat and is neutralized by *B. welchii* antitoxin. It can be precipitated with ammonium sulphate and a stable dry hæmolytic powder obtained. The filtrate from a young culture in peptone broth when given intravenously produces rapid lysis of cells with hæmoglobinæmia and hæmoglobinuria. Henry was of opinion that at least two inter-related toxic principles exist, a hæmotoxin and a myotoxin. The exact relationship between the hæmolysin and other toxins is not at present clear. Toxin affects smooth muscle (see p. 288).

Antitoxin.

Production of antitoxin. This is usually and conveniently carried out with horses; mules are quite satisfactory. Glenny and Allen tested some 50 normal horses during the war and found small amounts of antitoxin present in many of them. . More extensive investigations recently have confirmed this finding (Glenny, private communication). It is probably this natural immunity, together with the low toxicity of the toxin, that enables one to commence immunization in the average horse with either a small dose (e.g. 1 c.cm.) of toxin or living organisms (Weinberg, 1923). The doses of toxin, given intramuscularly weekly or bi-weekly, may be rapidly increased at short intervals for one to two months, when an antitoxic serum of satisfactory titre can be obtained. Some workers give the dead or living organisms intravenously and the toxin subcutaneously or intramuscularly.

Instead of the toxin preserved with 0·5 per cent. phenol often employed in serological laboratories, toxin kept at 37° C. after the addition of 0·1 to 0·3 per cent. of formalin until it has become atoxic to animals, i.e. toxoid ('anatoxin') may be used; Weinberg and Prévot (1924, 1925) describe the production of satisfactory amounts of antitoxin. These authors also use 'anaculture', i.e. a mixture of the bacilli and the broth medium which has been kept for 8 to 15 days at 37° C. after the addition of 0·3 to 0·5 per cent. of formalin; by this time the bacilli are dead and the culture atoxic to animals. There appears to be no clear evidence that the retention of the bodies of the dead bacilli in the antigen causes horses to produce antiserum more potent against either toxin or living culture than that produced by the injection of toxin.

Testing of antitoxin. The methods of testing and the units of measurement employed have been reviewed by Dalling, Glenny, Mason and O'Brien (1928). Bull (1917) and Bengtson (1920) used pigeons and injected toxin intramuscularly, the former based the unit of antitoxin on one lethal dose of toxin, the latter on ten doses. The Committee on Anaerobic Infections (Medical Research Committee, 1919, p. 120) based their unit on twice the dose of toxin lethal for the mouse when given intramuscularly. Weinberg (private communication, 1927) has used the intravenous method with rabbits, guinea-pigs and mice, basing his *B. welchii* unit on the amount of toxin, given intravenously, which kills a guinea-pig. For liquid toxin, this was approximately 0·5 c.cm., and a corresponding quantity of precipitated dried toxin was used. Later English work has mostly been done by intravenous injection into mice, a test dose of approximately 10 m.l.d. being used. Dalling and his co-workers (*v. supra*), until an official or international standard is fixed, propose to use as the unit the amount of antitoxin contained in 0·01 c.cm. of the serum used by Bengtson. One-tenth of this amount neutralizes between 10 and 20 m.l.d. of the toxin used by these authors. The toxin and antitoxin are mixed, the total volume being less than 1·0 c.cm., allowed to stand 1 hour at room temperature and injected intravenously into mice of from 18 to 20 gm. The average horse will produce serum containing from 50 to 300 of these ' units ' per c.cm. These authors further show that the same values can be arrived at for a series of antitoxins by either the intramuscular, intravenous or intradermic methods, once the proper dose of toxin for each method has been determined. They have investigated the relation of the content of antihæmolysin and of antitoxin obtained by the various methods and conclude that when using certain specimens of ordinary precipitated toxins the antitoxin values determined by intramuscular, intravenous or intradermic methods and the antihæmolytic value may show close agreement. Further investigation must determine whether this relationship holds good for all toxins and antitoxins.

The toxins of various anaerobes have been investigated to some extent by physiologists (Straub, 1919 ; Lautenschläger, 1920). Buttle and Trevan (1928) described the action on excised smooth muscle tissue of *B. welchii* toxin, and have investigated the quantitative relationships between toxin and antitoxin. They find that there is a reasonably close agreement between values so obtained and those got by the mouse-intravenous method.

Active immunization. Toxin to which antitoxin has been added until the mixture is just non-toxic to animals can be used as a prophylactic, and produces, a month after two doses of 1 to 5 c.cm. separated by a week, a degree of immunity in guinea-pigs which will enable them to withstand a certain lethal dose of living culture injected intramuscularly. There is little doubt that a mixture of antitoxin with toxin or toxoid would serve for active immunization of human beings if necessary. Weinberg and Prévot (1924) have used formalinized toxin ('anatoxin') for the production of active immunity in animals, and Weinberg and Ginsbourg (1927, p. 179) mention that 5 to 10 c.cm. of 'vaccin antiperfringens' has been used in man without harm, and suggest that this method may be of use if ever it were considered necessary to immunize human beings on the large scale.

Use of antitoxin in human medicine. In experimental work with animals it was found that antitoxin given a few hours in advance of the injection of cultures of *B. welchii* gave complete protection against general or local infection. If given after the animal had received culture or a lethal dose of toxin the point was soon reached at which no amount of antitoxin would save the animal. The results of prophylaxis and treatment during the war in the French and British Armies gave results that might have been predicted from the animal experiments. An adequate dose of the appropriate antitoxins injected immediately after the receipt of a wound appeared to give a degree of protection against the development of gas gangrene in the individual. Fairly large field experiments were carried out in the British Army in France, and those who watched the experimental work were, from the figures available, convinced of the value of the serum if given early after the receipt of the wound. In treatment the results were not so clear; the patient receiving a large dose of antitoxin at a time when the disease had not made great progress would be saved, whereas the one receiving antitoxin very late would, as under similar conditions in an attack of diphtheria or tetanus, die. The Committee of the Medical Research Committee (1919, p. 131) reported that 'In view of numerous factors which militated against success . . . we consider that the results obtained in France have exceeded expectations'.

When the serum is used in civil practice in connection with conditions caused by *B. welchii*, the animal experiments clearly indicate that antitoxin should be given as early as possible and in large doses. No sufficient data exist to decide what is an adequate dose; doses of 40 to 100 c.cm. of unconcentrated serum are mentioned by Williams (1926). It would appear to be wise, until further experience is gained, to err in the direction of over- rather than under-dosage and to give 50 to 100 c.cm. of unconcentrated serum or 40 to 60 c.cm. of concentrated antitoxin.

Lamb dysentery culture. Under this title, Dalling (1926; Gaiger and Dalling, 1923) has described a bacillus (or complex of associated bacilli of different species which he could not separate) obtained from 'lamb dysentery', a disease causing a mortality of from 10 to 60 per cent. amongst new-born lambs over wide areas in England and Scotland, and which probably exists in many parts of the world. The bacillus has a close relationship to *B. welchii*, many of the reactions of which it gives; an antitoxin made by the injection of the lamb dysentery toxin will protect against toxin or culture of *B. welchii*. The opposite relation does not hold, for antitoxin to *B. welchii* does not protect against toxin or culture of the lamb dysentery bacillus. These workers have, by immunizing the ewe so that the blood will contain a large amount of antitoxin just before lambing, shown that a large measure of protection can be given to the lamb. Antitoxin prepared in the usual way by immunization of horses, when given to the new-born lamb, gives also a high degree of protection against the disease. The general methods of production of toxin and antitoxin, titration, &c., closely resemble those relating to *B. welchii.*

B. ŒDEMATIS MALIGNI (VIBRION SEPTIQUE).

The preceding section with minor changes applies to this bacillus.

Toxin.

Peptone broth, Martin broth or trypsin digest broth containing preferably 0·5 per cent. glucose is used in deep columns with a layer of meat at the bottom, and the cultures allowed to grow at 37° C. for 36 to 48 hours. No special arrangements to secure anaerobiosis are necessary. Of an average liquid filtrate 0.025 to 0·05 c.cm. given intravenously to mice will kill over-night.

Testing of toxin. Subcutaneous, intramuscular, intravenous and intradermic methods have been used as well as the determination of the hæmolytic titre (Dalling, Glenny, Mason and O'Brien, 1928). Buttle and Trevan (1928) show that the toxin causes excised smooth muscle to contract; this action is neutralized by specific antitoxin and consistent titrations of antitoxin can be made by the method.

Antitoxin.

The general methods of production resemble those described for *B. welchii.* For the titration, the toxic filtrates from a 48-hour culture has been used intravenously by the English Committee (Medical Research Committee, 1919, p. 116), Weinberg (private communication), and Dalling, Glenny, Mason and O'Brien (1928). Weinberg based his unit on the amount of toxin which, given intravenously, kills a rabbit of 2 kgm. in an hour. The English workers use toxin precipitated with ammonium sulphate, of which approximately 0·4 mgm. will, when given intravenously, kill a mouse over-night. Their provisional 'unit' of antitoxin is that amount which will neutralize about 4 m.l.d. of a certain toxin. Average horse sera contain from 300 to 1,000 of these units.

Active immunization. Jensen (1906 to 1915) used dried culture and spore-bearing threads, but apparently finally adopted the use of dried serum *plus* dried serum-bouillon culture containing live spores for the prevention of 'bradsot' or 'braxy', a disease caused by *B. œdematis maligni.* Christiansen (1921) described these methods fully. Gaiger (1922) used the culture filtrate, freed from bacilli. Allen and Bosworth (1924) and Dalling (1925) used slightly underneutralized mixtures of toxin and antitoxin for protection against braxy in sheep. These latter mixtures gave a high degree of protection, as tested some weeks after immunization by the intravenous injection of toxin or the intramuscular injection of culture.

Use of antitoxin in human medicine. B. œdematis maligni antitoxin and *B. welchii* antitoxin are used in much the same type of case. Frequently the two organisms are present in wounds or in abdominal conditions; sera containing antitoxins to both these organisms were much used during the war, both prophylactically and therapeutically, and are now being used in civil surgery. Thus Weinberg (1927) states that serum containing antitoxins to *B. welchii, B. œdematis maligni, B. novyi,* &c., is injected as a routine prophylactic before laparotomy in many French hospitals.

B. NOVYI (B. ŒDEMATIENS).

The general methods of production and testing of toxin and antitoxin are again very similar to those relating to *B. welchii.* In the immunization of horses neutral mixtures are used at the commencement of immunization to produce a 'ground immunity' as in the case of tetanus. Toxin which, when given intramuscularly, kills a mouse in a dose of 0·001 c.cm. or less, can be prepared on the large scale. Antitoxin is usually titrated against the test dose (Weinberg, private communication), which contains about 100 m.l.d. Sera containing 20,000 of these units per c.cm. were produced during the war. Serum containing antitoxins for more than one organism appears to be desirable when wounds may be grossly contaminated, as during war. Multivalent sera were prepared during the war against *B. welchii, B. œdematis maligni* and *B. novyi* (Weinberg and Ginsbourg, 1927, p. 31), and in England (Medical Research Committee, 1919, p. 118), a serum containing the three above-mentioned antitoxins altogether with tetanus antitoxin was prepared in 1918. It is possible to make multivalent sera in each horse, but in practice it is found preferable to mix the sera of horses immunized with one of the antigens, or at most two.

B. CHAUVŒI.

This bacillus apparently does not infect man; it causes blackleg in animals, commonly in young cattle, much less frequently in sheep and pigs; the culture filtrate is innocuous to animals; it is probable that where toxic filtrates have been recorded in the literature, other anaerobes were present in the cultures used. It is difficult in the laboratory to maintain the virulence of the living culture.

The bacillus and its products have been widely used for prophylactic injection of cattle (Arloing, Cornevin and Thomas, 1880). Those most frequently employed fall into three groups:

(*a*) Preparations of the sporing culture or, more generally, the dried (Kitt, 1899) or dried and heated muscle (6 to 7 hours at 90° C.), from an infected calf. The commonest commercial prophylactics of this group are small pills for insertion under the skin, or silk cords or threads soaked in the infected muscle juice. It is probable that the immunity produced by these methods is due to the presence of a varying but very small number of living spores.

(*b*) This group consists of the so-called 'natural aggressin', which is the expressed and filtered juice from infected muscle. It is used either as liquid, dried, or in the form of small pills for injection.

(*c*) The filtrate of cultures (Nitta, 1918; Eichhorn, 1918; Gräub and Zschokke, 1920; Leclainche and Vallée, 1913, 1923) ('artificial aggressin') has been used in recent years in Japan, France and Switzerland and elsewhere. Arloing, Cornevin and Thomas (1880) first showed that culture filtrate immunized well. There is no clear evidence that vaccination by this method gives a lower degree of protection than the other methods. Since the material is free of living organisms, it lends itself to more precise control than the others above mentioned, and if the immunity produced proves to be satisfactory, it is probable that this method will eventually displace all others.

In order to make prophylactics of groups (*a*) and (*b*) young bovine animals are injected intramuscularly at many spots with live culture. The animals are killed when the local disease is well advanced, and the muscle or muscle juice obtained. For group (*c*) the bacillus is grown for four days in ordinary peptone broth of neutral reaction with a layer of autoclaved meat at the bottom. Allen and Bosworth (1924) describe an interesting 'double prophylactic' which contains filtrate of *B. chauvœi* and a slightly underneutralized mixture of *B. œdematis maligni* toxin and antitoxin. Two injections of this double prophylactic give to guinea-pigs and to sheep complete protection against the injection of virulent culture of both *B. chauvœi* and *B. œdematis maligni*, the two organisms which cause blackleg, the latter being the usual organism found in sheep in Britain, and both in cattle.

Weinberg and Prévot (1924), Allen and Bosworth (1924) and McEwen (1926) have worked with formalinized culture and filtrate. The method is of interest and full of promise (Weinberg), but has not yet been used on a very large scale.

Antiserum can be prepared by the injection of a series of increasing doses of filtrate into horses; this will protect guinea-pigs against lethal doses of culture. Antitoxin was prepared by Kitt (1899) and Leclainche and Vallée (1900). Though often used in experimental work the antiserum has not been employed much in the treatment of blackleg, though there is every reason to assume that if given sufficiently early and in adequate dose, it would be effective. Weinberg and Ginsbourg (1927, p. 31) suggest the use of a mixed serum containing antibodies to *B. chauvœi*, *B. œdematis maligni* and *B. welchii*.

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CHAPTER X. *BACILLUS TETANI.*

BY PAUL FILDES (LONDON HOSPITAL).

WITH SECTIONS BY
WILLIAM BULLOCH (LONDON HOSPITAL)
AND
R. A. O'BRIEN AND A. T. GLENNY
(WELLCOME PHYSIOLOGICAL RESEARCH LABORATORIES).

General Characters.

'*BACILLUS TETANI*' is the organism, first adequately described by Rosenbach, which is recognized as the cause of tetanus in man and animals. Other bacteria which do not cause tetanus but have otherwise similar characters are now included under the same species name, it being recognized that the power to produce tetanus is not a constant feature. As is the case with other bacterial groups the precise boundaries between *B. tetani* and somewhat similar bacteria which are endowed with separate species names are indefinite. The group of species to which reference is made are all anaerobic organisms which are widely distributed in soil and the intestines of animals. Their outstanding feature, which almost renders them worthy of a separate subgeneric name, is their capacity for developing a spherical spore at the end of the rod, especially when grown in a 'digest' medium. Some of the species in this group (p. 365) ferment carbohydrates but do not digest proteins markedly. Others have the opposite character, and between these extremes is found *B. tetani* itself.

Except the tetanus-producing strains of *B. tetani* none of the members of this group have any medical importance.

History.

BY W. BULLOCH.

Modern ideas with regard to the pathology of tetanus may be said to have started with the experimental observations of two Italians, Carle and Rattone (1884). A patient developed typical and fatal tetanus after scratching an acne pustule in the suprahyoid region. Two hours after his death, matter was obtained from the wound and injected into a series of 12 rabbits, 4 being inoculated into the sheath of the sciatic nerve, 6 into the muscles of the back and 2 into the spinal canal. With one exception all the animals developed a disease which Carle and Rattone regarded as tetanus. From the tetanic rabbits a further series of 4 rabbits were injected. Two of these, which were inoculated with emulsions of sciatic nerve, developed tetanus, whereas 2 injected with the blood of the tetanic rabbits remained well. No tetanic symptoms followed the injection of ordinary septic material in a series of 8 rabbits. Carle and Rattone had thus shown that matter from a tetanic wound is tetanogenous. A. Nicolaier (1884), working in Flügge's Hygienic Institute in Göttingen, reported the results following the injection of samples of earth into house-mice, guinea-pigs, rabbits and dogs. With the exception of the last-named animal, a disease resulted which was identical clinically with tetanus. The anatomical changes found after death were trivial, and a bacteriological examination of the local inflammatory exudate showed cocci and divers bacilli, among them some that were fine and slender and somewhat longer than Koch's bacillus of mouse septicæmia. Apparently the same bacilli were found in microscopic sections of the wall of the local lesion but they had no invasive tendency. Nicolaier attempted their cultivation but was only partially successful. Impure cultures were obtained in the depth of blood-serum medium at 37° C., and such cultures produced tetanus. Impure subcultures were carried on to the seventh generation. Nicolaier found that tetanogenous soil was rendered inert by heating at 190° C. for 1 hour. Watery extracts of tetanic earth produced tetanus after filtration through a fine-mesh gauze. Nicolaier made a large number of experiments, and of 88 mice or rabbits inoculated with earth 64 developed tetanus. Eighteen samples of earth were examined and 6 failed to produce tetanus. The samples were obtained from woods, streets, meadows and fields, and on vegetables and roots. From the fact that the 'tetanus bacilli' were not disseminated throughout the body Nicolaier inferred that they probably acted by producing a strychnine-like poison. J. Rosenbach (1886), the surgeon in Göttingen, made important advances in our knowledge. He inoculated guinea-pigs with fragments of tissue taken from the demarcation zone of the thigh in a case of tetanus following frostbite. The animals developed tetanus and Rosenbach successfully transferred the disease through 4 guinea-pigs and 11 mice in series. The symptoms produced were identical with those which Nicolaier had described as following the inoculation of earth. In the material from Rosenbach's human case there were many cocci and bacteria, large and small, and Rosenbach attempted unsuccessfully to obtain pure cultures. In the impure cultures he noted, however, the presence of 'bristle-like' bacilli which took the form of a 'pin or a drumstick'. Realizing that these were spores he attempted to get pure cultures by heating the impure mixture to 100° C. for 5 minutes, but did not succeed. C. Flügge (1886) described the tetanus bacillus as having terminal oval spores; and as Nicolaier himself does not mention spores at all it must remain open to doubt whether the 'tetanus bacilli' he described were in fact tetanus bacilli.

Kitasato (1889), working in Berlin, is generally credited with having obtained pure cultures of the tetanus bacillus. His method was to purify the tetanic material by heating it to 80° C. for $\frac{3}{4}$ to 1 hour. The heated material was then sown in gelatin plates in a special apparatus under a hydrogen atmosphere. With his pure cultures he produced typical tetanus in animals. After death the lesions found were trivial and the bacillus had a very limited distribution. He could not recover it from the body after death and suggested that it disappeared quickly after producing some very active chemical poison. In Bologna, Tizzoni, Cattani and Baquis (1890) claimed to have isolated tetanus bacilli from the internal organs of animals inoculated with pus from a human case of lockjaw. They were unsuccessful with Kitasato's technique. Knud Faber (1890), a Dane, using impure tetanus cultures, proved the toxic nature of tetanus by reproducing the disease with cultures freed by filtration of all formed elements. In the same year Behring and Kitasato (1890) published their classical paper on immunization against tetanus. They showed that the cell-free blood serum of animals inoculated with tetanus toxin neutralized the toxin so as to render it inert. This antitoxic action became the basis of the serotherapy of tetanus, which was soon made available for practice.

In succeeding years it became evident that the antitoxic treatment of tetanus was not all that had been hoped for, and it was disclosed particularly through the work of A. Marie (1897) and his co-workers, and of Ransom (1900), and of Meyer and Ransom (1903), that the failure was due to the localization of the toxin in the central nervous system. The demonstration of the toxic nature of tetanus also led Vaillard and Vincent (1891) to note that tetanus spores do not vegetate and produce toxin *in vivo* except under special conditions, which they attempted to disclose. In later years comparatively little was added to our knowledge of tetanus until Tulloch (1917) demonstrated a number of serological varieties of the bacillus.

W. B.

Morphology.

The typical sporing phase of the morphology of the tetanus-bacillus has earned the name ' drumstick ', the staff representing the body of the bacillus and the spherical padded head the spore. The following description (Fildes, 1925[1]) applies to the bacillus in artificial culture as stained by carbol fuchsin-methylene blue for demonstrating spores.

In the non-sporing condition the organism shows no characteristic feature. It is seen to be a bacillus with slightly rounded ends varying in width from 0·6 to 0·3μ. Its length is indeterminate from a few μ to over two diameters of the field. These long filaments are to be found in the youngest surface cultures, and there is seldom any indication of division into constituent bacilli, although after 24 hours this division nearly always takes place. Those bacilli which do not form spores gradually lose their affinity for aniline dyes, and in many cases become almost completely autolysed. The development of spores in some cultures takes place sparsely, and in others freely. A strain which forms spores readily usually does so on the second day of incubation, but on the third or fourth on media containing much unchanged protein. The feeble sporers may show only a few after 8 to 10 days' incubation.

The morphology of the spore depends upon its state of development. At first it is a slight oval enlargement of one end of the bacillus, not capable of retaining fuchsin. It is next found to stain deeply with fuchsin, to acquire gradually a spherical shape and expand to the typical ' drumstick '. During this process the intensity of the staining with fuchsin often becomes less. The remnants of the protoplasm of the bacillus which originally surrounded the spore vanish early except on the distal side, where a small cap often persists. The outline of the spores is usually darker and more sharply cut than of those of most other bacilli. They do not attain their mature size and shape without acquiring the fuchsin-retaining character, nor do they readily become free from the bacillus even in old cultures. When this does occur a fragment of the body sometimes remains attached to the spore, and the appearance is then reminiscent of *B. sphenoides* (p. 366). While the spore is developing, the body of the bacillus tends to lose its affinity for dyes, but it does not become autolysed to the same extent as the non-sporing bacillus. It generally remains short—about four diameters of the spore, but it may be three or four times longer.

The familiar description of the sporing bacillus with spherical terminal spore is undoubtedly to some extent idealistic, unless this description is retained for ' mature ' spores, and other forms are looked upon as ' immature '. Some cultures certainly tend to retain the oval shape or do not attain a true sphere, while other cultures produce bizarre or involution forms in which the spore may be obviously oval. The bacilli in such cultures often remain filamentous and spores are rare. These latter frequently retain a surrounding rim of protoplasm which stains blue, and thus appear unusually large, while ' dumb-bell ' types with two spores may be observed. Sometimes associated with dumb-bell types are long bacilli in which the fuchsin-retaining material of the spore is arranged as amorphous granules along the course of the filament.

The pyriform swellings described by early writers are not common under ordinary conditions of growth. They are pear-shaped or oval swellings at any point in the body of the bacillus, which do not retain fuchsin, but have an unusually strong affinity for methylene blue. When occurring at the end of the bacillus they are hardly distinguishable from the early stage of spore development, and it is possible that they represent an aberrant and abortive form of this phenomenon.

Motility. The tetanus bacillus when examined under anaerobic conditions is actively but not highly motile ; it, however, rapidly becomes non-motile under the action of the light used in observation. The movement is imparted by means of flagella (Kanthack and Connell, 1897 ; Votteler, 1898), which, when viewed by the dark ground method, are seen to be of extreme tenuity and coiled in a permanent spiral. These flagella are very numerous and are attached over the whole surface of the organism but not at the ends. During locomotion the flagella are directed backwards at an angle of about 45°, and appear to spin on their longitudinal axes. They are also capable of being swung bodily at different angles, and so affect the direction of movement. These phenomena are best observed in the water of condensation of 6 to 10 hours' surface cultures. Motility persists in the early stages of spore formation.

Non-motile varieties have been isolated by Bauer (1924). These appear to be confined to the serological group VI. No flagella can be observed.

Staining reactions. The body of the bacillus stains with any of the basic aniline colours, and the spore, when ripe, retains fuchsin. The organism is Gram-positive, but with the commonly used Jensen's modification this reaction is slight, usually only a few individuals in a young culture retaining the stain.

Cultivation.

ANAEROBIOSIS.

B. tetani requires for growth strict anaerobic conditions, or more accurately in the sense of Mansfield Clark (1928), a certain degree of reducing intensity in the medium in which it is growing. In so far as the reducing intensity of the medium is countered by atmospheric oxygen, this latter must be excluded the more completely when the reducing intensity of the medium is slight. This matter need not, however, be discussed in this place. The facts with regard to *B. tetani* are as follows : No growth on the surface of a medium will take place in pure culture unless oxygen is rigidly excluded. No growth will take place in a liquid medium (10 c.cm. in a 6 × $\frac{5}{8}$ in. test tube) in air unless the medium has considerable reducing intensity. Thus there will be none in peptone water or broth, but growth (some strains) in broth containing glucose or other reducing substances. In deep agar germination will start below the surface, and the reducing intensity of the culture will then increase and growth spread to the surface. Active growth can, of course, be obtained under ' aerobic ' conditions by the use of any of the familiar procedures (addition of cotton wool, particles of meat, other bacteria, &c.), which have the effect of inducing or maintaining a high reducing intensity.

OPTIMUM TEMPERATURE.

So far as is known the temperature range for growth of *B. tetani* is similar to that of most pathogenic bacteria. Growth in gelatin at 20 to 22° C. does not appear to be so ready with freshly isolated strains as is usually stated. Older strains, however, after some 10 to 14 days at this temperature, exhibit the ' fir-tree ' culture of Kitasato (1889) to a greater or less extent. It is doubtful whether growth will take place at all in a gelatin stab culture if the surface is not covered with vaseline.

pH RANGE.

According to Dernby and Allander (1921) growth takes place between pH 5·0 and 8·5, with an optimum about pH 7·0 to 7·6.

In tests carried out by the author in glucose broth, vegetation was not observed on the acid side of pH 6·4 or above 9·2. The optimum was more alkaline than stated by Dernby and Allander, namely pH 7·4 to 7·8.

NUTRITIVE REQUIREMENTS.

B. tetani will grow on any of the ordinary laboratory media but better on the ' richer ' media than on ordinary agar. Some strains will not grow in a simple peptone water. It has the power of growing satisfactorily on media broken down by putrefaction beyond the limit which will support other bacteria, thus Tulloch's exhausted medium will act to some extent as a selective enrichment medium (vide Vol. IX).

Reducing foodstuffs, such as glucose, have no marked nutritive advantage, but by virtue of their reducing intensity will permit growth under conditions which would otherwise inhibit it.

INHIBITION OF GROWTH.

Growth of *B. tetani* may be inhibited by the presence of other bacteria, but the conditions which lead to this are unknown. Bonome (1902) noted this fact with *B. coli* and stated that the inhibitory action was destroyed by heating the coli broth culture to 80° C. for 1 hour. Possibly this effect may be related in some cases to the production of oxidizing substances by the accompanying bacteria. It is observed in cultures in which there is no excessive acidity.

APPEARANCE OF CULTURES.

Surface cultures. When grown upon the surface of an agar slope the great majority of strains do not form discrete colonies but spread as an extremely fine film. The non-motile varieties, however, and occasionally a few old laboratory cultures, tend to form colonies with or without a film spreading therefrom. The colonies are somewhat variable in appearance but are generally based upon the ' medusa head ' type and are transparent in consistency.

The film growths (Fildes, 1925[1]) are highly characteristic. In order to observe them satisfactorily a slope must be used which has been kept till the apex is dry. The body of the film on agar is so tenuous that it can sometimes be hardly seen, but at the zone where the agar has become dry, ascending filaments of growth, sometimes roughly parallel but at others recurved and tangled, spread upwards almost on to the bare glass. These filaments are more conspicuous on a richer medium and after 2 or 3 days' growth. The spread of the film is so rapid that an inoculation into the condensation water will have spread to the apex in under 24 hours. It can progress at the rate of 1 cm. per hour. The film and filamentous edge is diagnostic of *B. tetani*.

Shake cultures. In well isolated shake cultures colonies appear as faint, cloudy masses of filaments with a generally radial arrangement (McIntosh, 1917; Adamson, 1919–20). The fluffy masses may sometimes show a more opaque core. According to Hall (1922) variants may develop as compact colonies. In more heavily inoculated tubes incubated aerobically, a diffuse growth appears below the limit of air penetration and usually with stimulation at the limiting zone. After 24 hours the growth also invades the ' aerobic ' zone and even the surface. A few gas bubbles appear.

Stab cultures. In agar a faint cloudy and fluffy growth spreads out horizontally from the needle tract. In gelatin at 20 to 22° C. covered with vaseline, a fine fluffy growth develops in 10 to 14 days, consisting of radial outgrowths from the needle track, these growths being less evident in the upper part of the stab (fir tree of Kitasato).

Broth. Cultures in broth frequently show no opacity until the second day. A general opacity then develops and is maintained for a long time, except in the case of type III (p. 306) strains. These latter in 3 or 4 days become quite clear, the growth falling to the bottom of the tube. Obvious gas-formation is not observed in liquid cultures (Durham's tube), but occasionally a few minute bubbles may appear on shaking.

Blood-agar shakes. Shake cultures containing heated blood show a green ring about 1 cm. from the surface when incubated aerobically; this later tends to darken. A similar darkening is sometimes seen in old agar cultures (Kitasato and Weyl, 1890).

Blackening. Coagulated protein substances added to media all undergo a slight degree of darkening, particularly brain medium. This effect is much increased by adding metallic iron to the medium (Hall, 1922). A few bubbles of gas usually appear in such media.

Smell. The smell of mixed cultures of *B. tetani* is often reminiscent of horse dung. The smell of pure cultures (liquid) is more ' cheesy ' and ' acid '.

ISOLATION.

Any of the ordinary methods for isolating sporing anaerobes may be used in the case of *B. tetani*, but the technique of Fildes (1925[1]) has obvious advantages. An enrichment culture is first made in Tulloch's medium (vide Vol. IX) and grown for 3 or 4 days. When a case of tetanus is under examination it is important to take as much tissue as possible, in fact it is better to excise the whole local lesion. Samples taken with swabs or pipettes are seldom satisfactory. The culture should be heated for 1½ hours at 65° C. in order to destroy aerobes, since some of these appear to inhibit *B. tetani*, and *B. proteus* would mask the film growth. A drop of the mixed culture 3 or 4 days old is transferred to the bottom of a sloped tube of Fildes' influenza medium (vide Vol. IX) so well dried that the apex of the slope is hard. After 24 hours at 37° C. a film growth with characteristic upper edge (p. 303) will be found in a positive case. Other bacteria will be confined to the lower part of the slope and so the tetanus bacilli may readily be transferred to another tube without contamination. It may be necessary to repeat the process of subculture in some cases to separate all contaminations.

Very rarely this method may fail to isolate tetanus bacilli which may be recognized morphologically in the mixed culture, because they do not grow as films. In such cases they may be isolated by inoculating the surface of several tubes in series in order to obtain isolated 'medusa head' colonies.

The most practical confirmatory tests to apply to a pure culture are the agglutination test (p. 306) for the species and the toxicity test (p. 332) for pathogenic action.

MAINTENANCE.

Pure cultures are most satisfactorily preserved by sealing in small capsules samples of a meat culture some 5 to 7 days old; under these circumstances the survival is probably indefinite. Unsealed cultures are also long-lived but have been observed to lose toxicity.

Biochemical Reactions.

Proteolysis. Gelatin is liquefied by all strains at 37° C. The toxic varieties act more rapidly in general than the non-toxic. The former liquefy in 4 to 7 days and some of the latter may take 3 weeks, the action continuing after growth has ceased. Coagulated serum becomes slowly more transparent and friable. Growth is poor. Cubes of coagulated egg-white are readily attacked (Achalme, 1902; Coleman and Meyer, 1926). Coagulated meat is disintegrated slowly, so that it can be broken up with a rod. Milk is an unsatisfactory medium and shows no change. Peptone solutions are digested slightly (Reddish and Rettger, 1924). Indole is formed by all strains. There is usually a slight production of visible gas in solid media but this is not conspicuous in liquid. This organism has, therefore, slight proteolytic activity.

Carbohydrate fermentation. No carbohydrates are fermented with sufficient activity to cause a change in indicators incorporated in the medium (Achalme, 1902; McIntosh, 1917). Fildes (1925[1]), however, found 3 strains which inconstantly produced sufficient acid from glucose to change the reaction from pH 7·4 to about 6·5. Reddish and Rettger (1924) state that glucose is consumed but to a slight degree. The saccharolytic activities are, therefore, trivial.

Serological Reactions.

Until the publication of Tulloch's first paper (1917) dealing with agglutination of *B. tetani*, references in the literature to serum antibodies were sparse and conflicting and need not be discussed. The work of Tulloch confirmed by Bauer (1924) and Fildes (1925[2]) disclosed the fact that tetanus bacilli are divisible into several serological types (Types I to VIII) between some of which there is a sharp distinction.

Rabbits are readily immunized with 3 intravenous injections of bacilli heated to 65° C. for $1\frac{1}{2}$ hours to deprive them of toxin. The size of the dose is unimportant and may consist of 1 to 3 whole slope cultures 24 hours old, suspended in saline. Titres up to 1/1,600 are easily obtained, especially in the case of Types I, II and V, but prolonged treatment may produce much higher titres. In such cases, however, which require frequent injections into the ear, sufficient local damage may be done to determine vegetation of the spores and loss of the animal. It is, therefore, safer to add a protective dose of antitoxin.

Agglutination may be carried out in the water-bath at 56° C., using an unwashed saline suspension of a slope culture, or a simple broth culture. Washed suspensions of Type III are spontaneously agglutinable. The suspensions may be heated to 65° C. for safety and 0·5 per cent. carbolic added. Agglutination takes place in most cases almost immediately in the lower dilutions and is of a particularly massive and flocculent quality. It is, however, important to remember when no agglutination is observed, that the non-motile strains found by Bauer (1924) agglutinate with difficulty and should be allowed to remain at least 5 hours in the water-bath. In these cases the aggregations are fine and granular.

Sera made from Types I, III and V are markedly specific to type even in a dilution 1/100, but Type II sera frequently agglutinate Type III also, but to a lower titre, and Type IV sera similarly agglutinate Type V (Fildes, 1925[2]). Little information is available about the 3 rare Types, VI, VII and VIII. The non-motile varieties already referred to appear to be confined to Type VI. In the case of group agglutination occurring, the correct type can be determined by the ordinary procedure of absorption of agglutinins. In order to obtain sharp results, it is, however, necessary to absorb the serum with two successive charges of suspension. Fildes (1925[2]) recommends for this purpose the use of a polyvalent serum made by immunization with a selection of all types.

Attention was drawn by Fildes (1927[1]) to the fact that the large floccular type of agglutination undergone by the motile film-producing strains and the granular agglutination of the non-motile strains, were akin to ' H ' and ' O ' types of agglutination observed among certain aerobic bacteria. Felix and Robertson (1928) proved that these two varieties closely imitated the behaviour of the aerobic bacteria, in that the two separate and distinct antigens ' H ' and ' O ' were found to exist and to evoke the production of H- and O-specific antibodies. These authors pointed out, no doubt correctly, that the division of strains of *B. tetani* into serological types depends upon observation of the ' H ' agglutination alone, the more feeble and slow aggregation of the ' O ' type being overlooked. In their opinion the ' H ' agglutination is ultra-specific, and differences do not necessarily indicate any important differences in the microbes. The ' O ' agglutination, however, corresponds in its incidence with complement-fixation and does not disclose serological differences in the group. This is in accord with the fact that there is no difference between the toxins elaborated by the various types disclosed by ' H ' agglutination.

Agglutination with monovalent or polyvalent serum is not confined to bacilli which are endowed with the power of producing tetanus toxin. Positive results are also frequently obtained with strains which are non-toxic but are otherwise identical with *B. tetani* (p. 333), and, further, positive results are also obtained with strains, usually non-toxic, which show tendencies to depart from the ' ideal ' morphology of a toxic strain. According to Fildes (1927[1]) all such agglutinable strains are to be looked upon as true tetanus bacilli in process of undergoing variation to a greater or less extent. For practical diagnostic purposes, therefore, the agglutination test may be relied upon to disclose the species of a suspected organism, and may most conveniently be applied by using a polyvalent serum made from all types.

The Morbid Effects produced by B. tetani.

The morbid effect known as 'tetanus' or 'lock-jaw', which, under certain conditions, follows infection with *B. tetani*, is due wholly to a soluble toxin of extreme potency which is secreted by the bacillus as it vegetates at the point of inoculation. Under natural conditions the infection is due to contamination of a wound with material containing tetanus spores. These spores are widely distributed in nature, particularly in soil (p. 321), and thus will frequently be carried into wounds; nevertheless tetanus is rare (p. 315). The explanation of this phenomenon is that, though the spores are carried into a wound, they seldom germinate and produce toxin. The conditions necessary for germination are not commonly attained. An analysis of these conditions will follow later (p. 327). Here it is enough to note that the infection is never pure, and that some degree of suppuration is invariable, although this may have become inconspicuous when the patient comes under observation. It is noteworthy that tetanic wounds are very frequently found to contain foreign bodies, in civil practice particularly splinters of wood.

Tetanus toxin is not only secreted *in vivo*, but also in cultures. These cultures will, therefore, produce tetanus experimentally, but the tetanic effect is solely due to toxin preformed *in vitro* and not to any bacilli contained in the culture. The bacilli are in pure culture incapable of vegetating *in vivo* (p. 328).

EXPERIMENTAL TETANUS.

The result of an inoculation of a toxic culture of *B. tetani* is similar in all mammals and in some birds and cold-blooded animals. The effect is best demonstrated with liquid cultures, filtered or unfiltered, using, in the case of a guinea-pig, from a fraction of a cubic centimetre to 1 c.cm., according to the toxicity of the strain.

Susceptibility of laboratory animals.

The susceptibility of the common experimental animals differs considerably. It is customary to give a table showing the relative amount of toxin required to kill one gramme of animal, but it should be understood that this information is only approximate and varies with the age of the animal—the younger are more susceptible—and with the route of inoculation. Table I is compiled from the literature, the figures for mice, guinea-pigs, rabbits and cats approximating to those found by the Author.

TABLE I.

Relative amount of toxin required to kill 1 *gm. of animal.*

Horse	1
Guinea-pig	2
Monkey	3
Mouse	4
Goat	6
Rabbit	24[1]
Dog	300
Cat	2,400

In man, general tetanus has been produced by a prick from a pipette merely moist with toxin (Nicolas, 1893).

According to earlier workers there is a constant relation between the smallest dose required to produce symptoms and the m.l.d. for different species of animals. Thus in the mouse the m.l.d. is only 3 times the smallest symptom-producing dose, while the guinea-pig requires 6 times and the rabbit 100 times. It is doubtful to what extent these figures are of value; certainly they do not apply to intravenous inoculation.

The minimal lethal dose. The minimal lethal dose in the same species of animal varies with the point of inoculation, being larger the more this is peripheral in relation to the central nervous system. Sawamura (1909) has shown that injection into the toes requires 4½ times more toxin than into the thigh muscles, while injection into the leg requires 50 per cent. more than into the thigh; when toxin is introduced directly into the sciatic nerve and still more so into the brain or cord, the minimal lethal dose is found to be much smaller than is required for intramuscular injection. According to Loewi and Meyer (1908) the m.l.d. in the fore limb in the rabbit is much smaller than in the hind limb. The minimal lethal dose also varies with the route of inoculation. The subcutaneous route requires rather more than the intramuscular (Sawamura, 1909) and the intravenous considerably more. According to Marie (1897) the intravenous m.l.d. is 7 times greater; the Author has found that in rabbits about 5 times the intramuscular is necessary. With regard to the accuracy of measurements of the m.l.d. it is necessary to point out that most authors do not mention whether the m.l.d. they describe is capable of killing all animals inevitably or only a proportion of animals. It will be noticed throughout this paper that far-reaching deductions are often drawn from results following inoculation of one or small multiples of the m.l.d. and it should always be borne in mind (without labouring the point further) that these multiples may not have been fatal doses in particular instances. When a dozen guinea-pigs are each inoculated with what is supposed to be 2 m.l.d. it often happens that some survive. This cannot be accounted for by differences in the exact site of inoculation but depends upon fluctuations in the susceptibility of different animals.

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[1] Following von Behring most German authors describe the rabbit as 150 times more resistant than the mouse, or as 600 in this table.

The incubation period.

An incubation period always intervenes between the injection and the onset of symptoms. The length of this interval varies with different animals, with the site of inoculation and the size of the dose. Table II, elaborated from Courmont (1926), gives average figures for different animals :

TABLE II.

Mouse	8–12 hours.
Guinea-pig	12–18 hours.
Rabbit	18–36 hours.
Cat	28–70 hours (Meyer and Ransom, 1903).
Monkey	30–36 hours (Sherrington, 1917).
Dog	36–46 hours.
Man	4 days (Nicolas, 1893). 5 days (Küster, 1905).
Ass	4 days.
Horse	5 days.

The more peripheral the injection, the longer the incubation. Sawamura (1909) gives 4 days for injection into the toe of rabbits, 3 days for the leg and 2 days for the thigh. After injection into nerves, cord or brain, the incubation period becomes progressively shorter, to a minimum of 1 hour after intracerebral injection in the guinea-pig (Ransom, 1900–1). Comparing similar multiples of the m.l.d. the period is longest after intravenous injection, and is also longer after subcutaneous than after intramuscular.

Signs of experimental tetanus.

The signs of experimental tetanus are usually described as following two orderly sequences, the so-called ascending and descending tetanus.

Ascending tetanus is the form commonly observed in small laboratory animals inoculated in a hind limb. The striking feature is a tonic unrelaxing spasm of the muscles of the inoculated limb (local tetanus), followed by a spread of relaxing spasms to the muscles of the other leg, trunk and fore limbs in an ascending direction. The characteristic of the descending type is that no tonic unrelaxing contraction (local tetanus) occurs in the region injected wherever the injection be made, and the subsequent course is marked by involvement of arms, trunk and legs downwards. This type is generally seen in the large experimental animals. It will appear later that ascending tetanus is due to absorption of toxin by the nerves in relation to the point of inoculation and subsequent spread through the nervous system, while descending tetanus is due to initial absorption by the lymph-vascular system and subsequent attachment by all peripheral motor nerves. The classical sequence of ascending tetanus is only observed when lymph absorption is minimal, that is, with intramuscular doses of toxin little above the m.l.d. If the injection is subcutaneous or larger than the m.l.d., absorption by the lymph is more marked, the classical ascending effect is interrupted, and the animal is attacked with the general spasms of the descending effect before the former has been fully elaborated.

Except when the amount of culture injected is large, no conspicuous lesion is found at the point of inoculation. When, however, the dose is such that death follows in under 24 hours and the toxin is fresh, a local œdema is found, often slightly blood-stained. If the injection is made into the thigh this œdema will involve the whole thigh, inguinal region, and the lower part of the belly wall (Fildes, 1925[2]). These inflammatory effects are, however, chiefly notable after injection of fresh whole cultures and are also produced by non-toxic strains. It is, therefore, more probable that they are due to the lytic poison (p. 335) than to the spasm-producing.

Ascending tetanus. When 1 or 2 m.l.d. are inoculated intramuscularly into the hind leg of a small animal (mouse, guinea-pig, rabbit, cat, dog, monkey), a period of incubation elapses during which no abnormal signs are observed. The first evidence of a disability is usually that the inoculated leg tends to slip backwards as the animal progresses. The animal is unable to right itself with the usual promptness when placed on its back. Later the limb is found slightly abducted and the ankle joint extended. In the rabbit the foot is supported on the toes and ankle clonus is present. The condition is one of spastic paresis. The leg becomes gradually more extended until it is quite stiff from spasm of opposing muscles, the toes are spread out and the sole directed upwards (local tetanus). The tail of a mouse becomes stiff and drawn over to one side, but that of a cat appears to be unaffected. The next stage in the progress of the disease is for the leg on the opposite side to become affected, but the rigidity is never comparable to that seen in the leg inoculated. Frequently, however, the opposite leg remains free and the sequence is taken up by the trunk muscles, which cause an arching or lateral flexion of the spine. Finally, the fore limbs become spastic so that the animal is abnormally raised on them. No obvious lock-jaw is observed. During this time, or often earlier, all muscles are in such a state of reflex irritability that the slightest stimulus will bring about a general spasm. The animal lies on its side. All limbs are extended, the head is drawn back and opisthotonus may be observed. These general spasms soon pass off except in the leg inoculated, but recur at ever decreasing intervals and last longer. The animal is cyanosed and unable to move. The rabbit usually dies in a spasm, but mice and guinea-pigs sometimes become flaccid before death. Apparently spontaneous general convulsions are more often seen in descending tetanus and probably do not occur in the pure ascending type.

This is the sequence of events in a guinea-pig or rabbit which dies between the fourth and seventh day, but when death occurs later, general convulsions are not conspicuous. The animal is spastic and enfeebled. It feels cold, and death seems largely to be due to inanition. Death at a still later date may follow the injection of toxin without any symptoms of tetanus at all. Tizzoni, Cattani and Baquis (1890) described this after injection of acid toxin, while Dönitz (1897) saw it in rabbits, but not in mice or guinea-pigs after mixtures of toxin and antitoxin not fully neutralized, or after minute doses of toxin. It is, however, also to be observed in guinea-pigs after injection of non-toxic cultures ; the animals waste gradually and die in about 3 weeks (*tetanus sine tetano*). This condition may be supposed not to be due to the spasm-producing toxin, but to other constituents. It is reminiscent of a similar condition in experimental diphtheria.

In the more commonly observed variety of mixed ascending and descending tetanus following a larger dose, the local tetanus is followed rapidly by general spasms which exhibit no noteworthy sequence. In the acutest cases death may occur in 18 to 20 hours, but with small doses the incubation period in guinea-pigs is about 36 hours with death on the third or fourth day. It is easy to produce a local tetanus with doses less than the m.l.d., without subsequent death. This may disappear after a few days or it may persist for weeks, in which case wasting of the limb is observed.

Although the ascending variety is the feature of experimental tetanus in small animals, it is nevertheless not unknown in horses, but the local affection is never of the extreme degree observed in small animals (Brunner, 1894 ; Courmont and Doyon, 1899 ; Basset, Monvoison and Pincemin, 1919). It may be remarked also that symptoms localized at first in the muscles of the inoculated limb were noticed in both recorded cases of inoculation tetanus in man (Nicolas, 1893 ; Küster, 1905).

Descending tetanus. This is the form of inoculation tetanus commonly observed in the horse. It is of interest because it resembles the disease as seen naturally in man. Wherever the inoculation is made, the first signs are seen in some particular group of muscles, usually of the head, more or less characteristic for the species of animal. The muscles first affected are not, however, subject to the unrelaxing spasm of ascending tetanus. In the horse the first sign is that the nictitating membrane, instead of remaining covered by the lid when the head is suddenly raised, falls over the cornea (Youatt, 1831). A lifting of the tail is also a very early sign. The subsequent course of the disease does not follow such an orderly sequence as in ascending tetanus, but in general it is descending in that the fore limbs are affected before the hind limbs. The course is more rapid and general convulsions more marked.

Although this form is not ordinarily observed in small laboratory animals after subcutaneous or intramuscular inoculation it is important to note that under special circumstances it may occur. Zupnik (1900) was the first to show this by inoculation in parts of the body free from muscle, namely, the dorsum of the foot, the tip of the tail or in the neighbourhood of joints. Sawamura (1909) also produced descending tetanus in rabbits by inoculation into the toes. Among the small laboratory animals descending tetanus is most commonly observed in rabbits owing to the ease of intravenous inoculation. The first sign observed is a disability in feeding due to a slight lock-jaw. The animal assists the process of mastication with its fore paws. The spread of the spasms to fore limbs, trunk and hind limbs is rapid. The animal stands with extended and stiff legs and convexly arched back. Later it is unable to stand and during a spasm the limbs are extended, the spine dorsiflexed and head and tail folded back upon the spine.

Special routes of inoculation.

Subcutaneous and intramuscular. The descriptions already given may be taken to be typical of intramuscular or subcutaneous inoculation in the leg. Since, however, the onset of symptoms is observed first in the muscles in relation to the point of inoculation, characteristic symptoms may be evolved by inoculation into single muscles or into different parts of the body, thus, distortions of the face (cephalic tetanus) with inoculation into the area of the facial nerve, or spasm of the orbicularis palpebrarum muscle after instillation into the conjunctiva (Brunner, 1892[1]).

Intravenous inoculation. In all animals the symptoms are similar to those described but are of the descending type. The interval between onset of symptoms and death is much shorter than after intramuscular injections, while the incubation period is longer and the m.l.d. larger.

Intraperitoneal inoculation. The first sign in this case is a lateral curvature of the spine followed rapidly by general tetanus.

Ingestion. Toxin introduced into the stomach produces no ill-effects (Tizzoni and Cattani, 1890; Thalmann, 1900), this being due to a destructive action of acid (Fermi and Celli, 1892; Rabinowitsch, 1907; Vincent, 1908[2]). Similarly, it is harmless when present in the gut (Thalmann, 1900). This may be due to destruction of toxin by intestinal bacteria (Vincent, 1908[1]) or by pancreatic juice and bile (Vincent, 1908[3]). On the other hand, Ransom (1898) ascribed the lack of effect not to destruction in the gut but to absence of absorption, but Breton and Petit (1908) were able to demonstrate antibodies in the blood after injection of toxin *per rectum*, and held that there must be a slight absorption from the gut. This view is strongly supported by TenBroeck and Bauer (1924), who fed guinea-pigs with tetanus spores and found that antitoxin and agglutinins appeared in the blood.

Inhalation. According to Thalmann (1900) neither toxin nor culture has any effect when inhaled, but if a catarrh is induced experimentally, then tetanus results. On the other hand, Küster (1905) mentions the case of a worker in von Behring's laboratory who twice developed attacks described as tetanus from inhaling the dust of powdered toxin. Subsequently the same man suffered from inoculation tetanus and recovered. The Author has been informed of a similar case which occurred in this country as a result of grinding dry toxin. The patient developed general tetanus but made a complete recovery.

Intracerebral. This produces a characteristic condition known as cerebral tetanus (Roux and Borrel, 1898). In 8 to 12 hours after inoculation into the substance of the brain of a quantity of toxin much less than the m.l.d., the rabbit becomes highly restless and jumps about in its cage as though in fear. Out of its cage, it stands on tiptoes and rushes about, colliding with obstacles and hiding in corners. Polyuria is marked. It then is attacked with epileptiform convulsions from which it recovers for short intervals. In acute cases death occurs in 12 to 20 hours, but a chronic form exists in which death is delayed for 14 days, with much wasting. Permin (1914) produced the same condition by inoculation into the vitreous to avoid local irritation of the brain. The disease in the guinea-pig (Ransom, 1900–1) is similar but the incubation period is only 1 hour and death follows in 4 to 5 hours. In the rat a dose larger than the m.l.d. is required. The mouse merely becomes stupefied and dies from wasting.

Injection into the posterior roots. Meyer and Ransom (1903) described a special form (tetanus dolorosus) which follows injection of toxin into the posterior roots. This is characterized by sudden spasmodic attacks of pain entirely localized in the skin areas supplied by the posterior root involved. The attacks are reflex and are occasioned by the slightest stimulus, as by blowing on the hyperæsthetic area or later on any part of the body (Fletcher, 1903). The animal, with a cry, bites at the affected part. The attacks last a few seconds only but soon recur. Death follows apparently from exhaustion without any signs of motor tetanus. Injection into the cord produces mixed motor and sensory tetanus.

Intrathecal. Toxin injected into the cerebro-spinal fluid passes rapidly into the blood stream and has the same action as after intravenous inoculation. If, however, a slight injury to the brain occurs, cerebral tetanus follows; or to the cord, mixed tetanus dolorosus and motor (Ransom, 1900–1).

Intraneural inoculation. As shown by Tizzoni and Cattani (1890) and many subsequent workers, toxin injected into a nerve, such as the sciatic, produces the same effect as injection into the muscles within the area of distribution of the nerve. The m.l.d. is, however, small and the incubation period short. Injection into a purely sensory nerve (posterior tibial in the dog) does not produce tetanus (Fröhlich and Meyer, 1916).

Experimental tetanus in birds and cold-blooded animals.

The large number of experiments carried out in this field have led to somewhat conflicting results. Most of the earlier workers were unable to tetanize birds. Using, however, very large doses, tetanus was produced in pigeons by Babès and Puscariu (1890) and Behring and Ransom (1898). It is said that the m.l.d. per gm. animal is 48,000 times that required for the horse. Hens were tetanized by Courmont and Doyon (1893[2]). They are even less susceptible (m.l.d. horse, × 360,000). The goose on the other hand is described as being more susceptible than either hen or pigeon (m.l.d. horse, × 12,000).

Metchnikoff failed to produce tetanus in a number of cold-blooded animals (p. 349). On the other hand, the frog can undoubtedly be tetanized, but only when its temperature has been raised to 25° C. (Babès and Puscariu, 1890; Courmont and Doyon, 1893[1]; Buschke and Oergel, 1893). One or two c.cm. of a potent toxin is necessary. At 25° C. the incubation period is 8 to 12 days; at 37° C., 4 days (Courmont and Doyon, 1898). The symptoms in the frog are described variously, this being due possibly to different routes of inoculation. After injection into the dorsal lymph sac the onset is general. The animal may be so stiff that it can be supported by the feet in a horizontal position (Gumprecht, 1895). On the other hand, Brunner (1894) states that the onset is in the fore limbs even when inoculated in the back. Zupnik (1905) produced ascending tetanus by inoculation into the dorsum of the foot, but this result could only be obtained by Fröhlich and Meyer (1916) by cord inoculation.

Birger and Rawitsch-Birger (1926) have described a spasm of the vessels of the isolated ear of the rabbit on infusion of toxin. This occurs only at a temperature above 19 to 22° C. Below this point no spasm occurs, which they attribute to a lack of fixation by the vascular endothelium. They suggest that the absence of tetanus in the cold frog is also due to a lack of fixation.

Experimental tetanus in man.

Two cases of experimental tetanus have been recorded in man (Nicolas, 1893; Küster, 1905), and another has come to the Author's knowledge. Nicolas was affected by pricking himself deeply in the hand with a syringe needle which had been used for filtered toxin. The incubation period was 3 days 15 hours, as shown by sudden spasm of the thumb on placing the hand into cold water. The onset was purely ascending and slow; trismus on seventh day; opisthotonos on thirteenth, together with the customary signs of general tetanus. He was treated with chloral only. Definite improvement on the twenty-fifth day; on the fifty-fifth complete recovery. The last sign to disappear was contraction of the hand. The case of Küster was a laboratory attendant who broke a flask of toxin while inserting the plug. He cut his hand and the fluid poured over the wound. Antitoxin was injected 2½ hours later, but local tetanus started 5 days later. He was treated with intraneural injections and the disease did not spread. This same man 7 and 5 years previously had attacks of tetanus from inhaling dried toxin. The Author has been told of a case in an English Serum Institute which was ascribed to the same cause. The patient recovered from generalized symptoms under antitoxin treatment. It will be noticed that no deaths have followed these accidents.

NATURAL TETANUS IN ANIMALS.

Tetanus has been recognized as occurring naturally in man and a relatively small number of animals. It is common in horses. According to Hutyra and Marek (1922) in the German Army between 1899 and 1913, there were 56 cases of tetanus among 95,000 horses. The mortality in Budapest from 1886 to 1917 was 79·9 per cent.; in the Prussian Army, 1881–1911, 64·8 per cent.; in the French Army, 1904–5, 66·8 per cent. Tetanus is less common in bovines, sheep and goats. In most of such cases the infection follows an operative procedure. In other animals cases have been described as follows: in dogs by Tuttle (1904–5), Hughes (1906), Duncan (1907), Wooldridge (1908), Wood (1910), and McCunn and Fildes (1927); in pigs by Taylor (1906), Forbes (1910) and Williams (1910); in camels by Rabagliati (1920), and less certainly in monkeys by Morice (1876). The symptoms appear always to be of the descending type. Birds and cold-blooded animals are never affected.

NATURAL TETANUS IN MAN.

Incidence.

Young (1927) has extracted from the Registrar General's figures for England and Wales the information that in 1901–9 the incidence of deaths in males was 9·7 per million living and in females 2·9, whereas in 1919–25 the figures were 6·5 and 1·9. This fall in death rate is, however, largely due to the marked decrease in tetanus neonatorum (p. 319) this being only one-third as frequent at the later period. Among persons over 4 years of age the average decrease in deaths was slight.

Symptoms.

The symptoms of tetanus in man are too well known to require description. They follow closely the sequence observed in the descending type of experimental animals. It is, however, important not to overlook the fact that the ascending type associated with tonic spasm of the regional muscles as in experimental animals is far from rare. Faber (1890) was the first to call special attention to this fact and was followed by Brunner (1894) and Walthard (1910). The latter author reviewed 12 consecutive cases of tetanus in one clinic and found evidence of local tetanus in 5, and in 86 of 225 cases collected from the literature. The cases of the following authors may be cited: Klemm (1896), Halban (1896), Heddaeus (1898), Stintzing (1898^2), Gibb (1899), Axhausen (1905), Noer (1906), Bigg (1908), Brandstein (1908), Esau (1910) and Schmerz (1912). From these it appears that before the jaw is involved or even without eventual involvement of the jaw, painful twitchings may be noticed in the muscles about an injury. Such muscular twitchings frequently, however, extend to spasm of larger muscle groups, and indeed, though less frequently, to complete rigidity of a limb persisting after recovery in precisely the same way as in experimental tetanus. The early symptoms, if not very pronounced, are often overlooked not only by the patient but also by the doctor. They are certainly more frequent than the figures quoted would suggest.

During the war the proportion of cases of local tetanus to general increased from 1 per cent. to 23 per cent., this being due to the prophylactic dose of antitoxin given after the injury (p. 364). There are many records of cases of local tetanus in immunized soldiers during this period ; thus of 3 cases of tetanus published at the same time from one hospital, 2 were local in immunized soldiers, and 1 general and not immunized (Burnett and Tulloch, 1917 ; Ritson, 1917).

'Cephalic' tetanus following injuries to the face is of interest in that it is of the local ascending type.

Portals of entry.

Although tetanus in the great majority of cases can be traced to infection of an obvious lesion of the skin, it is often noteworthy that the size of the wound is out of proportion to the magnitude of the effects. In fact, it is sometimes difficult to find a wound, and it is only by improved technique that some trivial lesions can be incriminated as the primary focus. This difficulty has led to the introduction of terms such as 'rheumatic' or 'idiopathic' to apply to those cases in which no external lesion is found, the suggestion being that the infection has occurred from inhalation or ingestion or otherwise. The case described by Kallmeyer (1892) illustrates the danger of this conclusion. The skin of the patient 'showed on most careful examination in the presence of surgical colleagues, no sign of injury except a thaler-sized bedsore on the right elbow'. Nevertheless, at autopsy an abscess was found in the left sole. 'In spite of repeated examination during life, it was only on the table at autopsy that a dark spot was found on the left sole which turned out to be the distal end of a splinter, 1 cm. long, the proximal end of which connected with the abscess' already found.

Where a definite lesion exists internally, although in communication with the outside as in the lung or intestine, there is no reason to suppose that infection cannot take place, but at the same time the evidence in favour of this view, though probably good, is not entirely above criticism. The following instances may be cited : tetanus complicating pneumonia with tetanus bacilli in the bronchial secretions (Carbone and Perrero, 1895) ; tetanus from coal dust containing the bacilli in the external auditory meatus (Racine and Bruns, 1903) ; tetanus ascribed to carious teeth, with bacilli in the tooth washings (Luckett, 1910) ; tetanus and abscess of the lung containing tetanus bacilli (Motzfeldt, 1912) ; tetanus and the bacilli in an infarcted lung (Burrows, 1917) ; tetanus and bacilli in a loop of gangrenous gut (Knoblauch, 1923).

It is well established in experimental animals (p. 329) that tetanus spores having gained entrance to the tissues, may, if conditions for germination are unsatisfactory, remain *in situ* or be carried about in leucocytes for a long time. These spores may localize in suitable lesions produced subsequently and there germinate and cause tetanus. This mechanism also has been suggested as a cause of idiopathic tetanus in man, but there appears to be no positive evidence that cases of this sort have occurred. The literature reviewed by Natonek (1913) does not contain an instance worthy of serious consideration. It may be remarked that a relatively large number of spores must be injected to obtain the result experimentally, and it seems improbable that such conditions could exist in nature. According to Vincent (1904) latent spores in the body may germinate under the influence of hyperpyrexia and produce ' spontaneous ' tetanus. There appears to have been little confirmation of this observation (cf. p. 348).

Although a lesion of any type may be the source of infection there are certain varieties which have special interest.

(1) In 1901, 63 cases of tetanus occurred in the United States after vaccination, although in 1900 there was only one case (McFarland, 1902). Isolated cases had occurred before (Toms, 1894), while a few have been published since (Scott, 1910 ; Silverstein, 1911). A case was described by Findlay and Findlay (1902) in this country.

The source of the infection has been much discussed. On the one hand infected lymph has been suspected and on the other extraneous infection of a vaccine pustule. With regard to the former view there is only a small amount of evidence of infection of lymph. McFarland (1902), for instance, mentioned only one instance, while Francis (1914) at a later date in America could find no spores in lymph. Carini (1904), however, found 5 out of 50 samples infected in Europe and the Author has found toxic spores in the only sample of raw scrapings he has tested. It is, however, probable that the American figures do not give a correct idea of the contamination of the samples examined. When spores and lymph, for instance, are injected subcutaneously into a guinea-pig, tetanus often does not follow because the lymph has insufficient ' debilitant ' action (p. 330). Further, if the lymph is cultivated in broth before injection, any toxin formed may be destroyed by acid produced by other contaminators (p. 334). On the other hand it is obvious that a suppurating lesion on the skin is very subject to infection by scratching with dirty finger-nails. Further, if the sore is covered with certain types of dressing (bunion plasters) made of felt (which often contains tetanus spores) the source of infection is obvious (cf. p. 319). This was so in Findlay and Findlay's case.

McFarland argued that since the incubation period of post-vaccination tetanus was 20 to 21 days, it suggested that germination took place during the suppurative stage of the vaccine lesion, and this was what might be expected if the lymph itself was infected. But a similar incubation period might be met with if the sore was infected by scratching. According to McFarland the American epidemic was largely confined to persons vaccinated with one brand of lymph and the manufacture was hurried owing to a sudden large demand. On the whole, therefore, it seems quite likely that this epidemic was due to badly manufactured lymph and that activation of the spores in it was assisted by the presence of other contaminating bacteria. Infected dressings or scratching, however, seem to be more probable explanations of the rare sporadic cases.

(2) The introduction of injections of gelatin ' sterilized ' by the local apothecary as a hæmostatic agent has led to numerous cases of tetanus, the bacilli being demonstrated in several of the samples (Kuhn, 1901 ; Lorenz, 1901 ; Gerulanos, 1901 ; Schmiedicke, 1902 ; Eigenbrodt, 1902 ; Levy and Bruns, 1902 ; Dieulafoy, 1903 ; Lop and Murat, 1903 ; Tuck, 1904 ; Heddaeus, 1908).

(3) The hypodermic introduction of drugs has led to tetanus, and it is perhaps surprising that these accidents are not more frequent. Osburne (1892) described a case due to morphine injection, but the most notorious examples are the 19 cases which occurred at Mulkowal in 1902 (Mulkowal, 1907 ; Simpson, 1907), following the injection of Haffkine's plague vaccine. The precise origin of the infection or intoxication in these cases was never proved. A similar absence of data exists in the 18 cases which followed the injection of diphtheria antitoxin in Italy (Siegert, 1901), but it may be assumed that the horses used were in the incubation stage of tetanus (p. 356).

The occurrence of tetanus after quinine injections has been known for many years in the tropics (Roberts, 1876). Anderson (1892) stated that doctors in Mauritius had become averse to the practice of this method. Quinine, like other chemicals (vide p. 330), has the power of causing the germination of any tetanus spores which may be injected with it or which may be circulating already in the animal's body. This fact was first demonstrated by Vincent (1904²) and confirmed by Semple (1911). The latter author was investigating the incidence of tetanus in India after quinine injections, and concluded that these were not due to faulty technique but to the activation by the quinine of spores already circulating in the body. Attention has, however, been directed already (p. 317) to the objection that the demonstration of this fact in animals requires very artificial conditions which could not occur in nature. It seems, therefore, an inadequate explanation for this phenomenon. Quinine tetanus appears to have been rare in modern times, but four cases occurred in Palestine in 1925 (Macqueen, 1927). In these, while the incubation periods were long (12, 28, 28 and 21 days), the deaths occurred within 72 hours of onset. This would imply that germination took place several days after the injection and thus that the spores were not introduced at the time, especially as the technique was beyond criticism and other persons inoculated at the same time escaped. The inoculation of quinine produces necrosis which involves the needle track, and in animals these are very difficult to keep sterile. It is, therefore, possible that some time after injection into the buttock, especially in an intestinal carrier, spores and other bacteria may find entry into the needle track and germinate.

(4) Tetanus has followed the use of unsterilized dressings in contact with wounds. Kirmisson (1914) used felt, afterwards found to contain tetanus spores, for padding the plaster jacket of a case of scoliosis with an open sore.

(5) Dolley (1905) found that 9 out of 12 wads from blank cartridges produced tetanus in rats. This investigation was carried out in view of the fact that during 1904–7, 325 cases of tetanus had followed blank cartridge accidents during 4th July celebrations in the United States (July, 1904–7). Schmidt (1921) also has found cartridges to be infected.

(6) In explanation of the historic death of Commodore Goodenough in the '*Pearl*' after an arrow wound in the New Hebrides, Ledantec (1890) showed that these arrows would produce tetanus in guinea-pigs.

(7) The occurrence of tetanus neonatorum from infection of the navel was formerly common, but was 'almost peculiar to infants of the filthy poor' (Hirsch, 1886). This description certainly applied to the inhabitants of the island of St. Kilda, off the Outer Hebrides, where for many years there was a mortality from tetanus of half the children born (Morgan, 1862). This state of affairs was soon suppressed by simple hygienic precautions (Turner, 1895). During the period 1901–9, in the mining county of Durham, 62 per cent. of the deaths from tetanus in males occurred in children under one year of age, whereas for the whole country the proportion was 26 per cent. In more recent years, however, the proportion of cases of tetanus neonatorum has fallen. In the whole country during 1919–25 the death rate under 5 years of age was only one-third of that at the earlier period (Young, 1927). According to the records of the London Hospital, no case of tetanus neonatorum has been admitted since 1907. These facts are, therefore, in agreement with Hirsch's opinion that the prime cause of tetanus neonatorum is ignorance and squalor.

(8) Catgut. Cases of post-operative tetanus are extremely rare, but instances are known in which several obviously connected cases have occurred. It is said to be most common after hysterectomy (Koch, 1898; Audebert, 1925). Stitch abscesses containing catgut are usually present, and in these tetanus bacilli are found (Dyke, 1927). It is supposed generally that the bacilli are introduced in the catgut, and this is no doubt correct, but has never been proved. The Author has examined the whole stock of catgut remaining after such a case with negative results. Indeed, he has never found tetanus bacilli in catgut nor in the raw 'runners' from which it is made.[1] This is perhaps surprising, considering that practically all catgut finished for surgical use is heavily contaminated with sporing anaerobes, and that tetanus spores are found in the gut of

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[1] During the epidemic recently investigated by Mackie (1928), one specimen of catgut finished for surgical use was, indeed, found to contain tetanus spores. It may be concluded that this success was due to an unusually gross contamination of the material, since no less than nine cases of post-operative tetanus occurred in one institution.

animals (p. 322). The probable explanation is that the 'runners' are small intestine, whereas the multiplication of anaerobes occurs in the large intestine. The 'runners' would therefore be, at most, very slightly infected. Further, it seems probable that most of the contamination of the catgut does not arise from the gut at all, but occurs during the process of manufacture.

Incubation period.

The incubation period of tetanus in man, dating from the time of injury, is somewhat variable owing to the varying time elapsing between implantation of the spores and germination. McFarland (1902) analysed 387 cases from the literature and found the seventh or eighth day to be the commonest for the onset of symptoms. Among the 505 cases of the American Civil War it was the eighth day (Barnes, Otis and Huntington, 1883). A prophylactic injection of antitoxin has the effect of considerably lengthening the incubation period (p. 364).

Mortality.

In judging the mortality in cases of tetanus it is necessary to take note only of unselected series of cases. The mortality disclosed by the collection of isolated observations is, of course, much too low owing to recovery being the novel feature which calls for publication.

The following figures refer to cases untreated by antitoxin:

	Cases	*Mortality per cent.*	
Guy's Hospital, 1825–57 ..	72	86·1	(Poland, 1857).
Bethanien Hospital, Berlin	127	83·5	(Rose, 1897).
London Hospital, 1878–1923	71	88·7	(Author).
Danish Hospitals	199	79·0	(Permin, 1914).
American Civil War ..	505	89·3	(Barnes, Otis and Huntington, 1883).
Franco-German War ..	350	90·0	(War, 1885).
The Great War	72	83·3	(Cummins, 1923).

The mortality in untreated cases, therefore, ranged between 79 and 90 per cent., but the highest figure, 97 per cent. in 325 cases, was observed during 4th July celebrations in the United States, and these mostly were treated (July, 1904–7).

It has long been recognized that the mortality is higher the shorter the incubation period. In Permin's series, when the incubation was 10 days or less, 95 per cent. died; over 10 days, 70 per cent. The same effect is shown by Bruce's (1920–1) statistics, although these deal with protected soldiers treated in England, that is, excluding the acuter cases left in France who would raise the mortality figure for the short incubation.

Incubation 7 days or under; mortality 58 per cent.
,, 8–14 ,, ,, ,, 49 ,,
,, 15–21 ,, ,, ,, 30 ,,

The length of the incubation period has, therefore, a marked influence on prognosis, and should be taken into account when determining the efficacy of treatment.

The Mechanism of Infection.

DISTRIBUTION OF *B. TETANI* IN NATURE.

It is generally stated and accepted that tetanus spores are to be found in earth according to the degree of ' cultivation ' of this earth, and that the risks of infection are, therefore, greater in highly cultivated localities than in uncultivated. This view may be looked upon as a surmise rather than an established fact, since the volume of published evidence upon the subject is trivial. In fact, it may be said that little is known about the distribution of tetanus spores in the soil or elsewhere. Such information as is available on the distribution of the bacilli is as follows:

Soil. It is generally assumed that in the soil the bacteria are present as spores, but the possibility of vegetation under certain circumstances has never been considered. Direct inoculation of earth was the method originally used by Nicolaier (1884) to produce tetanus in mice. He obtained 69 positive results from 140 inoculations. Similar methods were used by Beumer (1888), Ohlmüller and Goldschmidt (1887), Kitasato (1889) and Sanfelice (1893). In all, positive results were obtained 87 times from 270 samples. A quantity the size of a pea must be inoculated into a mouse, and a number of the animals naturally die of malignant œdema. If the earth is dried before inoculation, death from malignant œdema is much reduced, but so also are the ' takes ' from tetanus.

Bossano (1889) was the first to attempt a survey of the world distribution of tetanus spores. He inoculated 96 samples from 27 localities very widely separated and found 43 positive. Thiriar (1894) made the statement that no spores were present in the earth of the Congo.

In recent times in America, impure liquid cultures have been used for inoculation (Dubovsky and Meyer, 1922; Hall and Peterson, 1924; Damon and Payabal, 1926). The number of positive results by this method is small, no doubt owing to the destruction of toxin in mixed cultures.

Among 101 samples of earth from 12 counties in England and Scotland examined by the Author, 79 were from cultivated, arable and grazing lands and 57 strains of tetanus bacilli were isolated. On the other hand no positive results were obtained from 22 samples derived from waste lands chiefly in the Scottish Highlands. The number of spores in any sample seems to be relatively small, it being estimated that one contained 800 per gramme and another 500.

It would thus appear that authors who have examined specimens of earth have found tetanus bacilli in them in a considerable proportion of cases, the proportion depending upon the suitability of their technique. The only direct evidence of an absence of spores in uncultivated areas seems to be contained in the Author's comparison between the wilds of

Scotland and the fields of England. Beumer, it may be added, claimed to have found tetanus spores even in dune sand, but the Author has been unable to confirm this.

The presence of spores in the earth naturally leads to contamination of vegetables (strawberries; Rabinowitsch, 1907) and equally naturally to ingestion by cattle.

Animal fæces.

In the fæces of horses, bovines and guinea-pigs, tetanus bacilli have been found, but apparently not in rabbits.

Horses. By direct injection of dung or impure cultures, tetanus has been produced 30 times from 107 samples (Sanchez-Toledo and Veillon, 1890; Hoffmann, 1905; Choukévitch, 1911; Lukas, 1915; Noble, 1915). Fildes (1925[1]) isolated 34 strains from 200 different stable horses in London.

Bovines. Sanchez-Toledo and Veillon (1890), Joseph (1910) and Lukas (1915), obtained 12 positive results by inoculation of 15 samples. The Author has isolated 44 strains from 55 cows, but Noble (1915) could find none in 21 cows.

Guinea-pigs. Sanfelice (1893), by inoculation, succeeded 7 times with 23 samples and Noble (1915) once with a single sample. TenBroeck and Bauer (1924) isolated tetanus bacilli from 27 per cent. of their stock and the Author from 7 out of 24 animals.

Human fæces.

In the intestines of healthy humans the bacilli were first demonstrated by Pizzini (1898). This result was confirmed by Semple (1911). Tulloch (1919) found 5 civilian hospital patients in London positive out of 31, and 7 out of 21 soldiers returned from the Front. Large investigations into this subject have been made in China and America (TenBroeck and Bauer, 1922; Bauer, 1924). Bauer and Meyer (1926), in California, found 120 normal persons to be carriers out of 487 (24·6 per cent.). On the other hand, Fildes (1925[1]) found only 2 out of 200 London Hospital patients positive, and 46 others examined by the Author were negative. Only 1 was positive out of 52 Cambridge hospital patients, while among 31 normal persons, mostly undergraduates of Oxford, 5 were positive. In Germany, Buzello and Rahmel (1924) also obtained positive results by inoculation of mixed cultures, but their method did not allow of precise enumeration.

As a curiosity, Hall (1925) has found tetanus bacilli in saliva.

It is thus clear that spores are present in the gut of a large proportion of horses and cows, particularly in cows, and obviously they are ingested during grazing. On the other hand, some populations of humans are also heavily infected, whereas others are not.

VIABILITY IN NATURAL HABITATS.

Although there are no exact records it is probable that the spores of *B. tetani* may survive under suitable conditions for many years.

Von Eiselsberg (1888) has recorded that a splinter which produced tetanus in man was equally effective in a rabbit 2¼ years later. In their natural habitat—soil—spores are not, however, indefinitely viable. Vaillard and Vincent (1891) found in surface earth bacilli which they took to be tetanus bacilli (although they had not, of course, the advantage of identification by agglutination) but which were not toxic. They concluded that these had been modified by air and light, even as cultures in their hands became extremely aberrant on exposure in air to diffuse daylight. The effect of light was accurately determined by Wesbrook (1894). Spores were actually killed by direct sunlight in air in 18 days but were unaffected in the absence of air. McLeod and Gordon (1923) found that agar cultures were killed by diffuse daylight in 5 days, this being due to a production of H_2O_2 in the medium under the influence of light and air.

Thus it is clear that spores, at any rate on the surface of the soil or on roads, are subject to relatively rapid death or modification. Whether the high proportion of nontoxic variants among strains from earth described by Fildes (1925[2]) is due to this cause is not certain (p. 333). According to Fildes (1927[1]) these strains were variants of toxic strains and the samples of earth which contained them were not necessarily non-toxic, although the population of toxic strains in such samples might be unusually low.

The extinction of spores in the earth is without doubt prevented by the continual reinfection of the soil by the dejecta of animals (Vaillard and Vincent, 1891). In spite of statements to the contrary, there is no doubt that the spores when ingested pass the stomach without injury and multiply in the large intestine (Sormani, 1892 ; Noble, 1915). TenBroeck and Bauer (1924) have made many experiments on this subject. They selected 100 guinea-pigs without spores in their fæces and gave them one large feed of spores. After being kept in sterile cages and fed on sterile food and washed vegetables for four months, 74 were found to be carriers, 21 free and 5 dead. By estimating the number passed in the fæces and comparing with the number ingested it was obvious that great multiplication had taken place and, furthermore, both antitoxin and agglutinin developed in the serum of the animals. Whether the carrier state when developed becomes chronic or whether it may be transient is uncertain. TenBroeck and Bauer's experience suggests the former, but Noble (1915) inclined to the latter view, and the Author, on re-examination of 6 horses and 2 men who were carriers, was unable to find the bacilli on the second attempt.

The continued existence of tetanus spores in the soil is, therefore, dependent upon a replenishment from the dejecta of animals.

Resistance to heat and antiseptics.

As bearing upon the chance of infection being carried by the various vehicles already noticed, it is customary to note the powers of resistance of spores to destructive influences applied for the purpose of sterilization.

Heat. Killed by 5 minutes at 100° C. (Kitasato, 1889; Tizzoni, Cattani and Baquis, 1890).

Killed by 1 hour at 140° C.; killed by 20 minutes at 152° C. (Morax and Marie, 1902).

Regularly survives 20 minutes at 100° C., usually 40 minutes and occasionally 60 minutes (Smith, 1908).

Killed by 15 minutes at 100° C. (Francis, 1914).

Survives 40 minutes at 100° C. (Zeissler, 1922–3).

Killed by 26 to 35 minutes at 100° C. (Sessler, 1924).

The results of experiments of this sort obviously depend very largely upon experimental conditions. Furthermore, they are not quantitative, and purport to indicate a thermal death-point which kills all samples. Actually even at 80° C. (in saline, exposed to air) considerable destruction takes place in 1 hour, and in 24 hours a suspension of visible opacity may be sterile. For practical purposes, from common knowledge, 115° C. for 20 minutes is invariably fatal, and 100° C. for any reasonable time is insecure. In the sterilization of instruments, therefore, it is best to add a few drops of liquid carbolic acid to the boiling water and keep thus for 10 minutes.

Antiseptics. The reports on the action of carbolic acid and mercurial compounds are so unreliable, and so subject to error both in experiment and application, that they need not be mentioned. The oxidizing antiseptics are, however, on a different footing as shown by Moll (1920).

1 or 2 per cent. perhydrol kills in 24 hours.
30 ,, ,, ,, ,, 5 minutes.
0·4 ,, chlorine water kills in 5 minutes.
0·5 ,, iodine (in KI) kills in 80 minutes.
1·0 ,, iodine trichloride kills in 30 seconds.

RISKS OF INFECTION OR INGESTION.

Various considerations, though somewhat speculative in the absence of direct observations, are, nevertheless, of interest in regard to the risks of infection. It may be assumed that the factor which governs the infection of a wound is chance, but that the risks are not the same in every case. It is obvious that if no spores are in the vicinity of an individual he will run no risk of tetanus, or of becoming a carrier, but if his surroundings are contaminated he will less easily escape a chance infection. On the other hand, if two persons are subjected to the same locality risk, and one is more subject by habit or pursuit to injury, he is more likely to become infected with tetanus. Personal risks and locality risks may therefore be considered, but both are no doubt much intermingled.

Probably among the most important of the personal risks is the state of civilization or education of the invididual. The views of Hirsch have already been quoted and the probable effect of decreased squalor upon the decline of tetanus neonatorum. Filthy surroundings and neglect of a wound obviously increase the likelihood of infection. The latter of these two factors would appear to be the outstanding cause of tetanus in civilized countries, foreign bodies, often quite gross, being permitted to remain in a wound when their mere extraction would prevent the vegetation of the spores.

It has often been remarked that coloured races are more subject to tetanus than whites in the same locality, and Hirsch (1886) has ascribed this to differences in clothing leading to greater risk of injury. For instance, Waring (1856) published the following comparison :

London,	1850–4.	Tetanus deaths : Total deaths	= 1 : 3,075.
New York,	1819–34.	,, ,, ,, ,,	= 1 : 748.
Bombay,	1851–3.	,, ,, ,, ,,	= 1 : 46.

Even recently Cooper (1917) recorded 295 cases of tetanus in one hospital in Bombay from 1910–17, whereas in the London Hospital there were only 31 during that time. There is no reason to suppose that coloured races are more susceptible in the immunity sense than white, the incidence among the former being 0·24 per cent. of the wounded in the American Civil War (Barnes, Otis and Huntington, 1883), and among the latter 0·20 per cent. This high incidence in Bombay is, no doubt, not due to clothing so much as to squalid conditions, since cases of puerperal tetanus and tetanus neonatorum are also extremely frequent.

The prime factor in the incidence of tetanus is usually ascribed to the degree to which the land is cultivated, and, therefore, the degree to which it is contaminated with spores. If, for instance, the incidence among the wounded during the investment of San Sebastian in the Carlist War (Alcock, 1838) was 2·2 per cent. and during the three years of the Crimean War, 0·2 per cent. (Crimea, 1858), it is argued that San Sebastian is more cultivated than the Crimea. It is, however, doubtful to what extent this argument is justified. At Waterloo, tetanus was ' inconsiderable ' (Thomson, 1816), but in France and Belgium in the late war, the incidence was 0·9 per cent. of the wounded before prophylaxis was introduced (Bruce, 1920–1). During the whole war the incidence in France and Belgium (Cummins, 1923) was 0·147 per cent., but in other theatres about 0·01 per cent. and these included Italy.[1] While admitting that in Gallipoli, for instance, the spore content of the earth might be small, there is no reason to suppose that there is any essential difference in this respect between San Sebastian, Belgium or Italy. There is, however, information to show that conditions at San Sebastian and in Belgium were such as to lead to contamination of wounds more readily than in the Italian campaign. The former were very much more ' squalid ' than the latter.

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[1] The official records for the ' other theatres ' are unsatisfactory. Seven cases, for instance, occurred among 535 wounded in the Cameroons (Cameroons, 1921).

It seems, therefore, probable that it is not so much the degree of cultivation or the number of spores in the earth (assuming that there are some) which induces infection but the facility with which these spores are carried to the wound. Young (1927) has arrived at a similar conclusion in a study of the geographical incidence of tetanus in England. Although the Author has been unable to demonstrate any obvious difference in the counties from the point of view of spore content, Young has shown that there is an abnormal incidence of the disease in those counties in which arable farming is carried on (particularly in Cambridgeshire), and, therefore, in which the agricultural population is greatest, but in which there is no concentration of horses and the number of bovines is under the average. The fact of being a labourer on the soil would not appear to be essentially significant since the same geographical distribution is also observed in females ; but the important fact is, in the words of Young, ' the extent to which the soil is liable to be turned up '. It is obvious that there may be many spores in the soil under grass, but these are enclosed ; in ploughed areas, however, the spores are not only available on the surface, but are no doubt carried and distributed over all objects in the neighbourhood, to cause a higher incidence even among the females who are not agricultural labourers. At the same time, of course, the incidence among the males of the district is higher than among the females, because their occupation exposes them more frequently to injury.

The conditions governing infection of the gut are obscure. Different geographical areas show wide variations in the number of healthy carriers. Even in the same area at different times variations exist. Thus Tulloch found a considerably higher rate among hospital patients in London than did Fildes some years later. It is clear that the nature of the food must be important in this respect, a diet consisting largely of lightly cooked or uncooked vegetables being more likely to lead to infection. Tulloch's cases were no doubt largely vegetarian owing to the war, while the consumption of vegetables in Fildes' cases was slight. It is reputed that vegetable diets are in favour in China and America and this may account in part for the high carrier rate. Buzello and Rahmel's cases were also stated to be largely vegetarian. Further, as Pizzini (1898) indicated, a close association with spores (ostlers) is more likely to lead to infection, and among the 5 normal persons found positive by the Author (p. 322) 3 were in this category : namely, 1 bacteriologist working with tetanus, 1 protozoologist working with horse dung and 1 farm worker. It seems, therefore, that infection of the gut is largely a matter of the chances of carrying spores to the mouth, whether by food or otherwise.

THE BEARING OF SEROLOGICAL TYPES ON INFECTION.

It has been stated by TenBroeck and Bauer (p. 351) that a certain degree of immunity can follow the ingestion of tetanus spores, this immunity being specific to type. Under these circumstances, if ingestion of spores is common, the type most prevalent in the gut should be least prevalent in cases of tetanus. The following are the serological types isolated by the Author from different sources :

Type	*I*	*II*	*III*	*IV*	*V*
Cases of tetanus	7	1	8	0	4
Human fæces	4	1	0	0	3
Horse dung	8	5	7	0	14
Cow dung	3	2	10	0	29
Soil	3	4	5	6	30

From these results it would appear that outside man, Type V is decidedly most prevalent, but it is not most prevalent in cases of tetanus. If, therefore, the prevalence of Type V in England leads to its most frequent ingestion (the slight evidence shown indicates that it does not), it is in favour of TenBroeck and Bauer's view that this ingestion creates some small barrier to infection.

It is worthy of note that the incidence of the different serological types is not constant in all parts of the world :

Type	*I*	*II*	*III*	*V*	*IV*	*VI*	*VII*
Wounds received in France (Tulloch).	60	25	35	5	1	—	—
Fæces of man and animals in China (TenBroeck and Bauer).	21	17	52	1	155	10	5
Fæces in California (Bauer & Meyer).	78	9	21	3	6	0	—

Thus whereas results from France are similar to those from California, the Chinese are more nearly alike to those of the Author in England.

Conditions Determining the Germination of Spores.

In the words of Vaillard and Rouget (1892), tetanus spores ' have to germinate and vegetate before secreting the toxin which produces the disease '. These processes are entirely independent and the former has no relation to the pathogenicity or toxicity of the bacillus. Non-toxic strains of tetanus bacilli germinate under exactly the same conditions as toxic (Russell, 1927 ; Fildes, 1927[2]), but, of course, produce no disease. The vegetation of the bacilli is always transient, resporulation taking place after 24 to 48 hours' vegetation. During this time, only, is the toxin produced, and thus, if the amount secreted is small, the vegetation may have died out or the wound have healed before the signs of tetanus develop.

Lack of invasive power. As pointed out by Nicolaier and Kitasato, an outstanding feature of tetanus is the fact that the vegetating bacilli are confined to the point of inoculation and do not spread into the tissues or internal organs. Russell (1927) has studied the morbid anatomy of the local lesion in experimental tetanus produced in a ' natural ' manner by injection of spores and sterile earth. She found that although numbers of spores were removed into the surrounding tissues by phagocytes, the vegetating forms were very strictly confined to small areas in which necrosis of the tissues had taken place. At no stage of the infection was there any tendency for the vegetating forms to spread from these localized areas. The Author has also observed that if capillary tubes containing agar cultures of tetanus bacilli are implanted subcutaneously into guinea-pigs, the vegetation within the tube shows no tendency to spread from the open ends.

Nevertheless, a few undoubted instances are on record in which bacilli have been obtained after death from positions removed from the local lesion. Lacy and Murdock (1921–2) made positive cultures from the cerebrospinal fluid of a case of tetanus following gangrenous orchitis, but the Author has failed with the cerebrospinal fluid of three cases. Both Schnitzler (1893) and Porter and Richardson (1910) recovered the bacilli either by inoculation or culture from the inguinal glands of cases of gangrene of the foot. Reports by older workers of tetanus bacilli in the blood are open to objection. In experimental animals the results of several authors make it clear that cultures can be obtained frequently from the liver of the freshly killed animal, but only after a large dose, and if a large fragment is taken (Fildes, 1925[2]).

The statement that vegetating bacilli are strictly confined to the local lesion is not invalidated by these results as there is no evidence that in the positive cases the bacilli were vegetating. As shown later it is known that spores become passively disseminated and persist in certain areas for long periods. When vegetating forms are seen together with staphylococcus in suppurating glands as in Schnitzler's case, this is merely to be looked upon as a secondary local lesion.

Failure of vegetation of pure cultures. The important observation was made by Vaillard and Vincent (1891) that if a pure culture of *B. tetani* is injected into an animal, it dies of tetanus in due course, but the bacilli not only fail to multiply locally, but, indeed, rapidly disappear. This observation led to the conclusion that death from inoculation of cultures was not due to toxin elaborated *in vivo*, but to toxin preformed in the culture. If *B. tetani* was grown for a few days in gelatin at 22° C. no toxin was formed *in vitro*, and the culture of growing bacilli when injected was harmless. Similarly cultures deprived of preformed toxin by heat, though still containing viable spores, when injected had no effect. These observations have been generally confirmed and accepted, although some objection was made to the statement that heated spores did not produce tetanus. It was, however, shown by Vaillard and Rouget (1892) that the temperature—65° C.—first suggested was often insufficient, and that it was preferable to heat to 70 to 80° C. for 1 to 1½ hours (cf. p. 333). Fildes (1927[2]) on four occasions saw tetanus follow after spores had been heated to 65° C. for 1½ hours. In these cases the incubation was short as in ' toxin tetanus ' and the condition transient. It was concluded that a trace of toxin had escaped destruction in the thick suspension used, or that it had diffused from the bacillary bodies after heating (cf. p. 332). Thus Vaillard's main statement that pure cultures do not vegetate must be accepted as correct.

Fate of the pure culture in vivo. In an effort to account for the lack of vegetation of pure cultures, Vaillard and Vincent (1891) and Vaillard and Rouget (1892) studied the behaviour of spores on injection. Within 24 hours a large number of leucocytes were found in the local lesion and nearly all contained spores. After 2 or 3 days the degree of phagocytosis was so great that no free spores were left. The intracellular spores were pale and reduced in volume. These authors concluded that the spores were phagocytosed and digested and so could not germinate. At an early stage, many spores are also carried by phagocytes or perhaps by lymphatics away from the local lesion, and these may persist in various parts of the body for long periods. This persistence of spores has been studied by Vaillard and Rouget (1892), Tarozzi (1906), Canfora (1908) and Koser and McClelland (1918) with the result that cultures have been obtained from the liver as long as 3½ months after inoculation, although such long persistence is unusual. It is not known whether such persisting spores are free in the tissues or enclosed in phagocytes.

The relation of phagocytosis to germination. As already stated the striking appearance of the phagocytosis in the local lesion led Vaillard and his collaborators to conclude that this was a protective mechanism which led to the destruction of the spores. Anything which prevented or delayed phagocytosis permitted the germination of the spores. They designed a large number of experiments to support this idea. Thus spores would germinate: (1) in hæmorrhages and contusions because the advent of the leucocyte to the spore was delayed by such conditions, (2) in the presence of negative chemotactic substances such as lactic acid or quinine, (3) when mechanically protected by coverings of filter paper, (4) when other bacteria were present, because these injured the leucocytes or distracted their attention from the spores, (5) in hyperpyrexia because a leucopenia resulted.

In general, it may be said that Vaillard's experimental results have been frequently confirmed, but it is doubtful whether his conclusion that the presence or absence of germination depends entirely upon absence or presence of phagocytosis would be so readily accepted now as it was in the height of phagocytic enthusiasm. Several of the procedures noted above might, for instance, so alter the normal tissues as to provide a more satisfactory pabulum for growth (Tulloch, 1919). The organism might not grow in normal tissues. In this connection the action of Tulloch's exhausted medium may be recalled. On the other hand, as shown by Fildes (1927^2) a profuse growth may take place in a testicle of which the pedicle has been tied, before any histological evidence of degeneration has started. Thus a profuse growth may take place in practically normal tissues. It seems, therefore, doubtful whether any of these procedures stimulate growth by providing a better pabulum in the sense of a chemically broken down medium.

The particular lesions produced by the injection of chemical substances have been the subject of much study. Vaillard looked upon these as negatively chemotactic. All substances which have been used induce some degree, often a marked degree, of local inflammation and frequently of necrosis. Tulloch referred to them as ' tissue debilitants ', implying that the necrosis not only destroyed the local protective mechanism, including phagocytosis, but also supplied the satisfactory pabulum. Bullock and Cramer (1919) associated the ' kataphylactic ' behaviour of earth to an almost specific action of ionizable calcium salts, and ascribed the geographical distribution of tetanus to the varying content of the soil in these salts. From the work of Vincent (1904^2), Francis (1914) and Bullock and Cramer it is certain that the action of these substances is not upon the spores but upon the tissues. They produce a focus in which the spores can germinate, whereas they cannot germinate outside these foci. This is shown by the fact that germination will occur if the spores are injected into the focus 60 to 90 days after the introduction of the chemical, and also, though much less certainly, when the spores are injected into a different site. This phenomenon depends upon whether a wandering spore, probably in a leucocyte, happens to become involved in the chemical lesion. The germination takes place at the site of the chemical wherever this may be, not at the site of the spore injection. Francis appeared to remain in doubt as to the site of the toxin formation, but the Author has found that if spores are inoculated into one leg of a guinea-pig and calcium chloride into the other, local tetanus starts on the calcium chloride side where the vegetation may actually be observed. It is upon this undoubted experimental fact that the view is based that spontaneous tetanus in man may be due to the implantation of a wandering spore from a previous and forgotten injury into some internal lesion (p. 317).

As pointed out by Tulloch the activating effect of these chemicals is more pronounced in one species of animal than in another. Saponin is more certain in action in guinea-pigs than in mice and the reverse may be said of calcium chloride with equal doses, but with larger doses the effect is equally constant in the guinea-pig. There is some suggestion that there is a qualitative action ; thus trimethylamine produces more local disturbance in mice than calcium chloride, but its activating effect is not so great. It is certain that the value of an activator cannot be determined by the amount of local swelling it produces, but no observations have been made upon the amount of necrosis they cause. Russell (1927) has shown that calcium chloride or sterile earth in the guinea-pig produce a marked necrosis. Since no necrosis is produced by calcium chloride and citrate, the necrosis of calcium may, no doubt, be caused by the calcium ion, as held by Bullock and Cramer, but there seems to be no reason to suppose that the calcium chloride necrosis is any more efficient than a necrosis of the same size produced by any other agency.

In his experiments upon the effect of contaminating micro-organisms Vaillard isolated all the organisms he could from three cases of tetanus and inoculated them alone or together, with spores, into animals. He found that only one variety in five, for instance, had the activating effect. The Author has examined five cases of tetanus in this way and found only one culture which was effective in the guinea-pig. Vaillard found *B. prodigiosus* to be very active, while Friedländer's bacillus, staphylococcus, and *B. subtilis* were without action. The Author has found that a whole slope of staphylococcus will serve. On the whole, bacteria in pure culture or even in mixed culture are rather inconstant in action. According to Vaillard a strain may be positive in one animal species and negative in another, but a similar inconstancy may be observed in the same animal species.

Tulloch has called attention to the important part which *B. welchii* may play in tetanus. The toxin of this organism is a very effective activator. *B. welchii* was almost universal in tetanus during the war (but not so in civil tetanus), and it may be that when it is brought to germination its toxin may activate tetanus spores which otherwise may not have germinated. The toxin of *B. welchii* is more potent than that of vibrion septique.

With regard to the effect of hyperpyrexia in causing leucopenia and consequent germination, as described by Vincent, it may be said that this lacks confirmation. The question is referred to later (p. 348).

In general, the views of Vaillard and his collaborators, which have thus been summarized shortly, have been accepted. Fildes (1927[2]) only has found them unsatisfactory. According to his observations, although the amount of local phagocytosis is often (but not invariably) great, a considerable number of spores may escape phagocytosis and remain passive in the tissues, at any rate for several days. Vaillard and Rouget had already admitted the existence of free spores, but they ascribed these to artefacts due to rupture of loaded phagocytes in the preparation of the film. Fildes, however, disagreed with this explanation on the ground that the bodies of the bacilli were still attached to the spore, which was never the case when phagocytosis had occurred. Further, Russell (1927) has shown that free spores are found, though naturally with less certainty, in sections. Fildes, therefore, considered that phagocytosis obviously cannot account for the lack of germination of those spores which are not phagocytosed, although it no doubt accounts for the failure of the engulfed spores to germinate.

The oxygen tension of the tissues and the failure to germinate. In view of the fact that phagocytosis appeared to him to be an inadequate explanation for the failure to germinate, and that germination was sharply confined to areas of necrosis, Fildes (1927[2]) advanced the suggestion that the normal positive oxygen tension of the tissues would be sufficient to account for the lack of germination in healthy tissue. In necrotic areas, however, an obstruction would exist to the diffusion of oxygen from the capillaries, and thus these areas would become areas of asphyxia and fit for the germination of tetanus spores and further vegetation. Merely by ligation of the vessels of a cellular organ like the testis a more rapid and intense germination could be induced than by any other method. Even in two hours on the ligated side germination was in progress, while on the control side many of the spores were lying passively in the tissues under no apparent inhibitory influence. No histological evidence of necrosis was present at this time, and, therefore, gross alteration in the tissues was not an essential factor. According to Fildes, therefore, the lack of vegetation of spores in normal tissues is not due to any active immunological effort of the host, but is due to a lack of accommodation of the tissues of the host to the respiratory requirements of the anaerobic bacillus. In nature, according to this view, a slight inflammatory process is brought about by the combined action of bacteria of low virulence and foreign particles or bodies; this inflammation results in a vicious circle, i.e. some degree of capillary thrombosis and hindrance to oxygen diffusion, and increased local demand for oxygen by leucocytes and bacteria. These conditions induce local asphyxia and necrosis and the germination of any spores which may be present.

The Mechanism of Intoxication.

TOXIN.

The ordinary laboratory toxin consists of a filtered broth culture of tetanus bacilli. The poison is secreted by the bacilli and is detectable within 24 hours in cultures at 37° C., increasing in potency up to 7 to 14 days. There is little evidence that growth continues in ordinary broth after the first 48 hours, and, therefore, much of the toxin production can be looked upon not as a true secretion but as an autolytic phenomenon. The Author has observed a considerable production of toxin by heated 'spores' suspended in carbolic saline and maintained in the ice-chest (cf. p. 329).

Test for toxicity. For a routine toxicity test 1 c.cm. of a pure culture grown anaerobically for 4 days in a good liquid medium is injected intramuscularly into the thigh of a guinea-pig. In most cases the animal will die of acute tetanus in under 24 hours. The signs of this condition are so obvious that a control is superfluous in experienced hands, but in cases of doubt a second animal may be injected intraperitoneally with 500 units of antitoxin a few hours before the same dose of toxin is administered to it. This control animal will either remain in health or at most develop local tetanus if the toxin is highly potent. In the event of a strain showing no evidence of toxicity with this dose, a positive result may sometimes be obtained with a similar dose in a mouse. For the purpose of estimating the m.l.d., the culture may be used without filtration, since, as already stated, the bacilli in a pure culture do not develop toxin *in vivo*.

Variation in the Toxicity of Different Strains.

Tetanus toxin has sometimes such extreme potency as to rank it among the most fatal poisons known. It is common to find strains giving a toxin of which 0·0000002 c.cm. is fatal per gm. guinea-pig. On the other hand, some toxins are less potent and some strains indeed produce no demonstrable toxin at all. Thus Semple (1911) isolated one strain from earth which gave a toxin which was fatal in 0·25 c.cm., but the toxin of another produced only local tetanus with 2 c.cm. From 6 consecutive cases of tetanus in man, Fildes (1927[1]) isolated strains with m.l.d. in 4 cases 0·0001 c.cm., but in 2 no toxicity in 1 c.cm., although one exhibited slight toxicity when injected as spores with earth. The situation with regard to these totally non-toxic strains is at present confused. Several of the older workers have described such strains, but in the absence of toxicity it was then difficult to be certain that the culture in question was that of a tetanus bacillus. At the present time with the agglutination test this difficulty does not arise. Nevertheless there is clear evidence in the literature that non-toxic varieties can exist. Adamson (1919–20) records that he had an old strain from the Lister Institute which was always non-toxic, while Heller (1922) separated no less than 6 non-toxic variants from a standard Parke-Davis culture.

Since the introduction of the agglutination test a large number of proved varieties have been studied by Tulloch, TenBroeck and Bauer, and Fildes, with the curious result that Fildes (1925[2]) described a number of non-toxic strains which were consistently absent from the series of the other workers. Bauer and Meyer (1926) called particular attention to this. They found no non-toxic varieties among 129 strains from human fæces ; 2 required 0·1 c.cm. to kill a mouse, but the others were fatal in 0·01 to 0·001 c.cm. Fildes, on the other hand, found that 30 strains out of 75 from various sources had no demonstrable effect in doses of 1 c.cm. either upon guinea-pigs or mice. The Author has now examined 154 strains for toxicity and 78 have been non-toxic in 1 c.cm. doses in guinea-pigs. Only 1 out of 20 strains from cases of tetanus has been non-toxic. These strains, though perhaps more common among Type V, are distributed among all serological groups. They have been met with most frequently in soil.

According to Fildes (1927[1]) the non-toxic strains are variants from the ideal toxic strain and can be split off from these. Under certain conditions not established the toxic strain can become non-toxic spontaneously. The variation is not only manifest in the toxicity character but also in morphological and biochemical characters, but these variations do not necessarily run parallel. A non-toxic strain may have other characters normal, but on the whole they tend to abnormality more than do toxic strains.

Stability of Toxin.

Toxin in the form of broth cultures is unstable and readily loses its power of producing tetanus in animals. It is very susceptible to *heat.*

Faber (1890[2]) stated that it is destroyed at 65° C. in 5 minutes, and this has been generally confirmed. According to Kitasato (1891) it is rendered harmless by a temperature of 65° C. in 5 minutes, 60° C. in 20 minutes, and 55° C. in 1½ hours. On the other hand, under certain conditions, for instance when a thick suspension of spores is to be deprived of toxin by heat, a temperature of 65° C. for 5 minutes is quite insufficient. According to Vaillard and Rouget a considerable amount of the toxin is enclosed within the bacilli and only diffuses but slowly. Even after 1½ hours at 65° C. such a suspension may sometimes give evidence of being toxic (pp. 328, 332).

At room temperature toxin loses it potency gradually, particularly when exposed to light. The destructive action may readily be due to the formation of peroxides in the broth under the action of light. Sunlight destroys it in 18 hours (Kitasato, 1891), and it is well known that oxidizing substances convert it rapidly into a non-toxic condition.

The destructive effect of light is much increased by the addition of photodynamic substances such as eosin. Tappeiner and Jodlbauer (1904) found that 0·05 per cent. eosin caused destruction of toxin in diffused daylight in 3 days, while Huber (1905) stated that 1 m.l.d. was destroyed by 1 : 1,000 eosin in diffused daylight in 4 hours. According to Noguchi (1907) the injection of eosin *in vivo* will protect against tetanus. Rats infected with spore-containing threads will not develop tetanus if 0·2 to 0·4 c.cm. of a 2 per cent. solution of eosin is injected locally, although vegetation takes place. This observation is surprising and has not been confirmed.

For purposes of preservation the liquid toxin is precipitated with ammonium sulphate and desiccated. It is then highly stable and resistant to heat (p. 354). These dry toxins are, however, hygroscopic, and thus errors in weighing are liable to occur. It is usual, therefore, to dissolve the powder in distilled water and add an equal volume of neutral glycerin. MacConkey (1923–4) found these glycerinated toxins stable, and even a plain liquid toxin mixed with an equal part of glycerin was stable for 10 months. The Author has had less satisfactory results with glycerinated liquid toxins, but the glycerinated dry toxins are largely used and found to be stable.

Kitasato (1891) showed that *acid* had a highly destructive action on toxin and to this action is due the fact that large doses of toxin can be consumed *per os* without ill-effect.

The fact that other microbes growing in toxin broth may have a destructive action on the toxin and so render a mixed culture non-toxic although it contains highly toxic strains, has not been sufficiently appreciated by workers who have used such cultures for the recognition of tetanus bacilli. The point is well illustrated by Tulloch (1919). This observer determined in three instances the presence of *B. tetani* in mixed cultures made from wounds, although these cultures did not produce tetanus when inoculated into animals in the ordinary way. Moreover, it was found on inoculating a known toxigenic pure culture of *B. tetani* into tubes of a suitable medium, and then 'super-inoculating' such cultures with the mixed growths under consideration, that the resulting cultures were non-toxic. The tetanus bacilli present in the original mixed cultures were none the less pathogenic, for, if the cultures were enriched, so far as *B. tetani* was concerned, by growth under special conditions, and were then washed, the cultures so prepared, on being inoculated into animals along with a tissue debilitant, gave rise to tetanus.

Tetanolysin.

Ehrlich (1898), in the discussion on a paper by Kossel, mentioned that he had found a second toxic substance in tetanus toxin. This was 'tetanolysin', which dissolved many varieties of red blood corpuscles, but particularly those of horses and rabbits. Tetanolysin is extremely labile, and is rapidly destroyed at room temperature or in 20 minutes at 50° C. It may be absorbed specifically from toxin with red blood corpuscles, leaving the spasmin unaffected. An antilysin which differs from antitoxin may be produced (Madsen, 1899). The differentiation of toxin and lysin has been recently reinvestigated by Fleming (1927). Tetanolysin is only demonstrable satisfactorily in fresh whole cultures owing to its lability and to the fact that it is absorbed by filters (Reymann, 1927). Recent investigations by Neill (1926) show that lysin is produced within the first 24 hours of incubation of the culture, and is not destroyed by prolonged incubation. It is completely inactivated by heating for 10 minutes at 65° C. The lability of the lysin on keeping is due to its oxidation to an inactive product on exposure to air. This change is complete in 11 hours at 37° C., and is slower in the ice-box. The inactive oxidized product may be reactivated by reduction with sodium hydrosulphite ($Na_2S_2O_4$), but not the heated lysin.

Tetanolysin has been supposed to be the agent in toxin which sometimes leads to death from wasting without spasms, but Miyamoto (1900) has produced this result with toxins free from lysin, and, therefore, some other constituent may be involved in this condition.

Lysin is produced by non-toxic strains as well as toxic. Its pathological importance is uncertain, but it is probably the cause of the blood-stained œdema which is frequently seen in guinea-pigs after injection of fresh whole cultures. Vaillard and Vincent (1891) ascribed to toxin a leucocyte repellent action, but Tulloch (1919) showed that this could not be due to the spasm-producing fraction. On the other hand, the latter author demonstrated that young cultures contained substances which interfered with phagocytosis, and that these disappeared on storage. The Author has also observed a leucotoxic action under conditions similar to those described by Vaillard and Vincent and also with non-toxic cultures. It therefore seems probable that tetanolysin, but not spasmin, has leucotoxic activity.

EXPERIMENTAL INTOXICATION.

A very large number of papers have been published upon the mechanism by which toxin exerts its morbid effect. It has, of course, always been recognized that the effect of the toxin was upon the nervous system, and this view was soon supported by the demonstration of toxin almost exclusively in the nervous tissues of cases of tetanus. It may be said that it is generally accepted and has been so accepted for some years that toxin exerts its effect on the nervous system by reason of the fact that it is absorbed by peripheral motor nerves ; that it ' flows ' up the axis-cylinder of these nerves to exert some deleterious effect on the anterior horn cell and other cells higher in the central nervous system. In the following pages are given the main observations on which this view is based. With regard to this work in general it is justifiable to suggest that much of it was probing, with great experimental difficulty, into matters upon which knowledge was then and is even now elementary, and, therefore, that it is unlikely to contain more than the nucleus of a correct idea.

Distribution of Toxin in the Body in Tetanus and Experimental Tetanus.

Blood and lymph.

Toxin has been demonstrated in the blood of man by Nissen (1891), Schnitzler (1893) and Permin (1914). In cases of tetanus in man this depends chiefly upon the acuteness of the case. When the onset is early toxin may be demonstrable, but when late any toxin which was previously present in the blood has already disappeared. Ransom (1900^1) showed that after intravenous injection, toxin passes rapidly into the lymph, being demonstrable in a sample collected from the thoracic duct during the first 15 minutes. An equilibrium is established between the two fluids at the twenty-sixth hour. In a similar experiment, but using very large doses, Teale and Embleton (1919–20) found the toxin in the first five minutes. After subcutaneous injection, toxin passes rapidly into the lymph and blood. This conclusion might follow from Kitasato's (1892) experiments with mice, in which he found that if the inoculated tail was not amputated within one hour, the animal would succumb. Ransom (1901) opened the thoracic duct of dogs after subcutaneous injection of toxin and found it in the issuing lymph for the first time after 30 minutes, though not in the blood till 1 hour had elapsed. The concentration both in the lymph and blood increased gradually, but at 5 to 6 hours it was still much higher in the lymph than in the blood.

After a variable interval the toxin is no longer demonstrable in the chyle or blood, but there appears to be some evidence that it persists longer in the guinea-pig than in the rabbit. According to Camara Pestana (1891), the blood of the guinea-pig is toxic in the acute stage, but on the other hand, in the rabbit, Marie (1897) could detect toxin for 7 hours only. Teale and Embleton, with subcutaneous injection into rabbits, found the chyle positive 8, 12 and 25 hours after, but negative

48 hours after. The blood was negative 6½ hours after inoculation, positive at 8 hours and negative again at 48 hours. In a cat the chyle became positive for the first time in 30 to 45 minutes.

After intramuscular injection toxin appears more rapidly in the blood (Marie and Morax, 1902, 15 minutes; Tiberti, 1905, 10 minutes). Marie (1897) had already pointed out that toxin was only slowly liberated from the subcutaneous tissues, and persisted longer in the blood in consequence.

In experiments on sheep infected by implantation of a tetanic splinter in the tail, McClintock and Hutchings (1913) found that the blood was toxic 3 or 4 days before the onset of symptoms. From the third to the seventh day (date of onset) the concentration increased and then diminished till death on the thirteenth day. Even after no toxin can be demonstrated in the blood or lymph the regional lymphatic glands still contain it (Teale and Embleton).

It is thus well established that toxin is absorbed from the subcutaneous tissues by the lymphatics and passes by the thoracic duct into the blood stream in about 30 minutes. In the absence of a continuous supply, it disappears from the blood and lymph after about two days, at any rate in the rabbit.

The organs.

According to Marie (1897) the toxin which disappears from the circulation has become fixed upon the various tissues and rendered inert. In his view the many authors who have described toxin in the organs have only disclosed it in the blood of these organs. When toxin is no longer present in the blood, the organs are free. In the case of the central nervous system it is certain that a fixation of this sort takes place (p. 339), but a definite concentration in other organs when the blood and lymph were negative has been noted in one instance only by Teale and Embleton (1919–20) in the case of the spleen. Neither Pettersson (1911) nor Wadsworth and Vories (1921) could find evidence of destruction of toxin or combination with it by leucocytes, nor could Permin (1914) by blood. Although, therefore, there is little evidence of absorption or destruction of toxin by the organs, it appears nevertheless probable that this must take place, since the m.l.d. intravenously is considerably greater than the subcutaneous m.l.d., and the difference must be destroyed or fixed during its circulation.

Urine, &c. Bruschettini (1892) described toxin in the urine of two tetanus patients, but this observation has not been confirmed (Stern, 1892; Brunner, 1892²; Blumenthal, 1896). The same author and Brunner (1892¹) also found toxin in the urine of experimental animals, though Tiberti, and Teale and Embleton failed. It would appear that early workers were misled by toxic effects of normal urine in large doses in small animals.

Brunner (1892²) claimed to have found toxin in the saliva (pilocarpin) of a rabbit after a large dose, and even in the saliva of a case of tetanus, but the possibility of spores having been present was not, of course, recognized. Stern found no toxin in the milk of a case of tetanus.

There is, thus, little evidence that toxin is excreted from the body in a toxic form.

The nervous system.

Cerebrospinal fluid. According to Stintzing (1898) toxin is present in the cerebrospinal fluid of cases of tetanus. Permin (1914), using large doses in mice, found it 3 times in 5 cases. These findings could not be confirmed by Blumenthal and Jacob (1898) or by Teale and Embleton. Ransom (1901) was unable to find toxin in the cerebrospinal fluid of experimental animals even after intravenous injection of large doses. Teale and Embleton were equally unsuccessful. It appears, therefore, that toxin injected does not penetrate to the fluid in detectable quantities, but conditions in natural tetanus may be such that this may take place.

Nerve trunks. With regard to the solid structures of the nervous system, there is little doubt but that after subcutaneous injection the peripheral nerves in anatomical relation to the point of inoculation always contain toxin at some time and occasionally the corresponding nerves on the opposite side (Marie and Morax, 1902; Meyer and Ransom, 1903; Tiberti, 1905; Teale and Embleton, 1919–20). This is the case even when the blood and surrounding tissues are free from toxin. The success of the demonstration depends, of course, upon the amount of toxin injected and the part of the nerve examined. Sawamura (1909) could find no toxin in the sciatic of a rabbit after injection of 1 m.l.d. into the leg, but always after injection of 2 to 5 m.l.d. With rather smaller doses it could only be demonstrated in the peripheral part of the nerve. Odier (1904) also pointed out that the examination must be made at a particular time after injection to be successful. Permin (1914) always found toxin in the peripheral part of the nerve, but it was only present in small quantities in the central part after large doses. He ascribed this result to the rapidity with which the toxin passed up the nerves, so that at any one moment the amount present was trivial.

Investigations of the nerves of human cases have been few. Brunner (1893) found toxin in the facial nerve in a case of cephalic tetanus, but Albert (1918) always failed to find toxin in the nerves of cases after death.

It has been mentioned already that the toxin which is absorbed from the site of inoculation by the lymphatics and passed to the blood is supposed to be absorbed from the blood by the peripheral nerves of the whole body and so to occasion the descending form of natural or experimental tetanus. Unfortunately, support for this view by demonstration of toxin in other than the local nerves is largely lacking. The only positive claim is by Morax and Marie (1903), who found toxin after intramuscular injection in all nerves, motor, sensory and sympathetic.

But the blood, cerebrospinal fluid and even the aqueous humour was also toxic, and, therefore, it is not conclusive that the toxin found in the nerves was not merely present in the tissue fluids (Fletcher, 1903). On the other hand, absence of positive evidence cannot be taken as being fatal to the view advanced, because relatively large quantities of toxin must be present in a nerve in order to be demonstrable by inoculation of the emulsified nerve into a mouse, and when it is remembered, calculating from Sawamura, that after injection of about 400 mouse m.l.d. into a rabbit's leg not one mouse m.l.d. is present in the sciatic, it is obvious that toxin diluted by the whole blood volume could not be detected in distant peripheral nerves. Furthermore, another difficulty arises from the fact that some of the toxin which is present in a nerve is masked in the animal experiment by being lightly combined with some constituent of the nerve. It is true that this effect is very slight with nerve trunks (Sawamura, 1909), and according to Asakawa (1898), Marie and Morax (1902) and Tiberti (1905), hardly demonstrable, but with brain tissue the effect is pronounced (Wassermann and Takaki, 1898).

The Wassermann-Takaki phenomenon. This observation, which has been confirmed generally, was that suspensions of brain tissue mixed with toxin are quite non-toxic when injected into animals, whereas liver, kidney and spleen have no such effect. The authors also stated that if the brain suspension was injected 24 hours before the toxin, the same protection was observed, or even if injected 4 to 6 hours after. The latter observations have not been confirmed. Wassermann (1898) represented, undoubtedly erroneously, that the effect of the brain suspension was due to antitoxin in it—sessile receptors in the sense of Ehrlich—and that his work demonstrated for the first time the source of antitoxin as normal fractions of cells thrown into the circulation.

Marie (1898) and Asakawa (1898) failed to get protection with brain previously injected, and the former also failed when the injections were made at the same time but in a different place. He showed that the action was due to a direct combination *in vitro* so that no toxin remained in the supernatant fluid after centrifuging (Knorr, 1898 ; Milchner, 1898) ; but the combination did not lead to any destruction of toxin, as would antitoxin, because it could be recovered again by extraction of the dried residue with water or by digestion with papain (Marie and Tiffeneau, 1908). This fixation of toxin by the brain is thought by some to be due to lipoidal substances in the brain and by others to albuminous. Landsteiner and Botteri (1906) stated that an alcoholic extract of human brain contained a fixing substance identified as 'cerebron'. Takaki (1908) confirmed this result, but found little cerebron in grey matter although this fixed better than white. On the other hand, Marie and Tiffeneau (1908) could find no fixation with ether extracts of brain nor with the various lipoids used by Landsteiner and Botteri. Laroche and Grigaut (1911) failed also. Both Marie and Tiffeneau, and Laroche and Grigaut, were inclined to incriminate protein substances as the fixing agent.

The neutralizing action of the nervous tissues is much less marked in the cord. It is greater in the grey matter than in the white (Marie, 1898) and, no doubt, resides in the nerve cells rather than in the fibres.

Brain and cord. In view of this phenomenon it is clear that toxin is apt to be overlooked by the method of injection of suspensions of brain into mice. Whether this is the reason or not there is no record of toxin having been found in the brain, unless inoculated directly into it (Ransom, 1900–1 ; Marie and Morax, 1902). Even then Ransom found that a considerable amount was not recoverable owing to fixation by the brain, and more was found in the cord, although the injection was intracerebral.

In the cord, toxin can be demonstrated more readily, especially after injection into a nerve trunk. After subcutaneous injection in the leg Bruschettini (1890) found toxin in the lumbar cord but not in the medulla ; after subdural cerebral injection it was present in the bulb but not in the lumbar region. By using a special method of desiccation and subsequent extraction, Marie and Tiffeneau (1908) were able to demonstrate toxin in the bulb after intramuscular injection. Toxin has also been found by Blumenthal (1896) in the cord of a case of tetanus, although the blood was negative. The claim of Shakespeare (1887) in the case of a tetanic horse is more dubious.

From this record it will be seen that there is fairly satisfactory evidence of a special concentration of toxin in the periphery of nerves in relation to the point of inoculation and that this concentration diminishes centralwards. Whether the diminution and eventual absence is due to real absence of toxin or to its fixation *in vivo* or *in vitro* in the course of the test, is unknown. There is no evidence of toxin in nerves unrelated to the point of inoculation, but evidence of this could hardly be expected.

The Method of Transmission of Toxin to the Central Nervous System.

The presence of toxin in the nerve trunks is a clear indication of the portal by which it gains access to the central nervous system, and that this presence alone is sufficient to produce the signs of tetanus is shown by the results of intraneural injections. Tizzoni and Cattani (1890), Marie (1897), Meyer and Ransom (1903) and Tiberti (1905), have all demonstrated that the same signs of local and general tetanus as are produced by subcutaneous injection, may also follow injection into the regional nerve under conditions which preclude any possibility of local leakage of toxin. The disease as evoked by this method only differs in being more severe, or following from a dose which would be ineffective on subcutaneous inoculation.

Toxin is supposed to enter the nerve through the nerve endings on the strength of an experiment by Marie and Morax (1902) and Tiberti (1905) :

If the sciatic nerve of a guinea-pig is cut as high up as possible and then toxin injected into the calf, it will be found in the whole nerve below the point of division. If, however, the nerve is cut at the knee and toxin injected as before, no toxin will be found in the part above the cut, although this part will still retain its vascular and lymphatic connections. (Actually some toxin will be found owing to some absorption by unsevered collateral nerves.)

The existence of the tòxin in the nerve substance rather than in its blood-vessels is also supported by Permin's (1914) observation :

If an injection is made intramuscularly into a passively immunized rabbit, local or ascending tetanus can be produced according to the dose. Since the blood and lymph is highly antitoxic in such a case, the toxin must have been absorbed by the nerve tissues.

The rate of absorption by the nerves is rapid. According to Marie and Morax, toxin is found in the sciatic 60 minutes after injection into the leg. Tiberti gives 1½ hours. Permin (1914) found that section of the nerve 1 hour after injection prevented the onset of tetanus and after 5 hours largely prevented it, but later little effect was produced.

The flow of toxin up the nerves is deduced from experiments of different type :

If toxin is injected into the legs of several guinea-pigs and the sciatics divided in all at a time when these should contain toxin, the toxicity of the central end will be found to diminish gradually until it is entirely lost in about 3 hours (Marie and Morax, 1902). Marie (1897) cut the second cervical nerve close to its emergence, and, after the wound had healed, injected one fatal dose into the paralysed paw. This dose had no effect, presumably because the direct nerve path being cut off, the toxin distributed by the blood was in a concentration too small to be effective upon the other nerves. Experiments of this type are not, however, always successful owing to absorption by collateral nerves, but if instead of merely cutting the sciatic, the leg is thoroughly enervated, then 2 m.l.d. are always without effect (Sawamura, 1909 ; Permin, 1914).

Meyer and Ransom (1903) took two cats and in one divided the spinal cord in the lumbar region. Both sciatic nerves of each animal were then injected with toxin, and after onset of local tetanus in the hind legs, antitoxin was given subcutaneously to neutralize the toxin in the blood-lymphatic system. The cat in which the cord had been divided remained *in statu quo*, while in the control animal the disease progressed to general tetanus and death. Tiberti confirmed this experiment.

If these experiments may be taken as direct evidence of absorption and transmission of toxin by nerves, there is indirect evidence that the nerve route is the exclusive channel of entry of the toxin. First, that toxin cannot penetrate nerve tissues by way of the blood-lymphatic stream, is shown by the fact that ' cerebral tetanus ' does not follow after intravenous injection. Secondly, a rabbit had antitoxin injected into the sciatic nerve on one side and toxin subcutaneously into each leg. Local tetanus developed on the unprotected side and the animal died, but its protected limb remained practically unaffected (Meyer and Ransom). Access to the lower centres for this limb was still open through the blood, and even through the nervous system from above, but intoxication did not occur when the peripheral nerve was blocked. Thirdly, antitoxin was injected into each sciatic nerve of a cat and then toxin administered intravenously (Meyer and Ransom). The animal died of general tetanus but the hind limbs remained quite unaffected. Tiberti, Permin, and Teale and Embleton confirmed these results in general, but sometimes found that the antitoxin did not protect completely. This, no doubt, is again due to the existence of collateral nerve filaments.

These experiments all indicate that if the nerve path is cut off, toxin has no access to the central nervous system, and this is the only evidence upon which the view is based, that even when the toxin is absorbed from the local lesion by the lymphatics it ultimately enters the nervous system by way of the peripheral nerves of the whole body.

As already stated, Morax and Marie (1903) found that toxin, after intramuscular injection, was present not only in the motor nerves but also in the sensory and sympathetic. They deduced from this that the latter were equally responsible with the motor nerves for the transport of toxin to the central nervous system. Meyer and Ransom, however, came to the conclusion that the toxin gained access by the motor nerves *only*. They based this view upon the discovery that if toxin is injected into the sensory posterior roots a form of tetanus results—tetanus *dolorosus* (p. 313)—which differs conspicuously from the ordinary motor type. Since a pure motor tetanus is always observed when the injection is into the nerve trunk, they claimed that the toxin must pass by the motor root exclusively. This conclusion is strongly supported by Fletcher (1903). He prepared a ' sensory ' sciatic nerve in a cat (and dog) by section of the anterior nerve roots. After five days' degeneration toxin was injected into the sciatic trunk without producing tetanus, but tetanus dolorosus followed rapidly after injection into the posterior root. Fletcher held that motor tetanus after subcutaneous injection is prevented by section of the anterior roots, but that the absence of dolorous tetanus in such a case is not due to blocking by the ganglion, but to the fact that the toxin does not ascend the sensory fibres. He pointed out that the peripheral sensory fibre is dendritic, while the central sensory fibre and the motor fibres are axons and endowed with the power of conveying toxin as shown by the effects of injection of toxin into them. He thus not only held that the absorption and conveyance of toxin was by the motor fibres but exclusively by them. This view is supported by Fröhlich and Meyer (1916), who injected toxin into the purely sensory post-tibial nerve of a dog without producing tetanus.

As already mentioned, the actual path in the nerve which serves to pass the toxin is generally stated to be the axis cylinder on the strength of the following experiment: The sciatic nerve on one side is cut high up in a number of animals and the wounds closed. Toxin is then injected into each calf of one of the animals at increasing intervals after section. The nerve is then removed from each side and they are compared for toxicity. Toxin is found in nerves cut 2 days before injection but not in nerves cut 10 to 15 or 30 days before. Thus the integrity of the axis cylinder is necessary for transmission (Marie and Morax, 1902; Tiberti, 1905).

A great deal of discussion has raged round the question whether the toxin flows in the protoplasmic axis cylinder or in the 'nerve lymph', but the discussion is confused by a lack of precise knowledge of the fluid paths in the nerves. Most workers have been governed by the results of Key and Retzius, who claimed to have demonstrated by injection that the perineural spaces are in direct communication with the subarachnoid space. This view may, however, be said to be abandoned; certainly Robertson (1916) and Teale and Embleton (1919–20) could only demonstrate a connection between the perineural spaces and the anterior roots and cord. There is no doubt that there are spaces in the nerves, presumably proper lymph spaces, in which toxin can percolate without affecting the nervous system. Authors repeatedly note that the injection 'without resistance' into a nerve may fail to produce tetanus. Fletcher (1903) injected into one of the right posterior roots producing right-sided dolorous *tetanus, and found that the toxin had spread into the posterior roots of the* other side without producing dolorous tetanus. The toxin on the left side *must, therefore, have been situated differently with regard to the nerve* tissue than on the right. These proper lymph channels would, however, be entirely distinct from the perineural tissue spaces possibly within the neurilemma, and it seems that the objections which have been raised to the 'lymph' theory could not apply to these spaces. Thus the division experiments of Marie and Morax and Tiberti mentioned above would lead to occlusion of these spaces and obstruction to flow of toxin, and the objections of Meyer and Ransom and others that the exclusively motor distribution is inexplicable on a lymph conveyance are also met by supposing a percolation through the perineural spaces, since these would have the same topographical distribution as the axons themselves. Furthermore, there is clear evidence that the toxin ascends not only to the anterior horn cells but progressively up the cord, which would entail a passage of gaps at the synaptic junctions if the toxin was actually carried in a protoplasmic stream in the axon.

Some direct support for the view of spread in the perineural spaces is afforded by Teale and Embleton. Seeking to block the perineural spaces in rabbits by inducing a slight inflammation in them, they injected intraneurally tincture of iodine, normal horse serum (controlled on the other side by normal rabbit serum) and egg-white controlled by saline. None of these procedures affected the functions of the nerves and so presumably the axis cylinders remained intact. Nevertheless, when toxin was injected subcutaneously into each leg, the onset of tetanus in the test limb was slower and the course milder than in the control limb.

Theories as to the Causation of the Phenomena observed in Tetanus.

The causation of the contractions.

The precise nature of the effect on the nervous system of the toxin which ascends the peripheral nerves cannot be said to be proved, but there seems to be no reason to doubt that changes are induced in or about the anterior horn cells or analogous cells in the bulb, which lead to an excessive or disordered susceptibility of the lower motor neuron to afferent tonic or sensory impulses (Gumprecht, 1895 ; Meyer and Ransom, 1903). The main experiments which have led to the view of the origin of the contraction in the anterior horn cell are as follows : The suggestion that the effect of toxin was upon the muscles themselves was disproved by the action of curare and deep anæthesia (Brunner, 1892[1] ; Courmont and Doyon, 1893[3]). In both cases the contractions are abolished except those of a fully developed local tetanus. The action of the toxin must be on the central ends of the nerves, since section of these stops the contractions (Tizzoni and Cattani, 1890), but only if the enervation has been thoroughly performed (Tiberti ; Pochhammer, 1909). The contractions are progressively stopped from above downwards by destroying the cord in this direction (Vaillard and Vincent, 1891), but they are not stopped by section of the cord (Meyer and Ransom). Thus the anterior horn cells must be intact and the upper motor neuron takes no part in the production of the tonic spasm.

A number of investigations upon the action of afferent stimuli have been made. According to some, section of the posterior root stops the contractions (Autokratow, 1892 ; Courmont and Doyon, 1893[3]), but others deny this (Brunner, 1894 ; Gumprecht, 1895). Permin (1914) investigated this point in dogs. First he divided the sacral and lumbar posterior roots on one side and allowed the animals to recover from the shock ; then he injected antitoxin intravenously to prevent general tetanus and so allow a more pronounced local tetanus to develop after intramuscular injection of toxin. By this means he showed that no result followed the toxin on the operated side, though local tetanus resulted from injection of toxin on the control side. He suggested that failure to divide all the posterior roots might account for Gumprecht's result. Roaf and Sherrington (1906) claimed that the contractions were due to a disorganization of the normal stimulatory-inhibitory reflexes. They found that stimulation of that part of the cerebral cortex which normally leads to contraction of the depressors of the jaw and inhibition of the elevators, i.e. opening, in the tetanic animal leads to closing by a stimulation of both sets of muscles and the resultant action of the stronger. In this view, assuming an excessive excitability of the anterior horn cells, afferent stimuli, instead of causing balanced inhibitions and stimulations, would cause stimulations only and contraction of all muscles.

The evidence is, therefore, in favour of the essential lesion being a disturbance in the reflex arc coupling the sensory and motor apparatus in the cord. In the view of some, this disturbance consists of an excessive susceptibility to afferent stimuli whether merely tonic or tactile; Roaf and Sherrington, on the other hand, claim to have proved not so much an excessive susceptibility as a disordered response to afferent stimuli.

Most authors consider that the unrelaxing contraction of the muscles observed in local tetanus differs essentially in origin from the reflex contractions of the other muscles. On the other hand, in the course of its development the local contraction is intermittent and as much subject to tactile stimuli as the reflex. In the local form the concentration of toxin about the nerve cells is, of course, very much greater than in the general, and may readily be supposed to cause such excessive or disordered excitability that the normal tonic impulses lead to continuous and violent stimulation. After the condition has persisted for a short time the situation is undoubtedly different, since changes actually take place in the muscles themselves, leading to a shortening and inability to relax. Such muscles differ from the normal side on inspection, and if the contraction persists for a long time wasting results. It may be supposed that changes of this sort are merely due to exhaustion, although Fröhlich and Meyer (1916) maintain the opposite, that it is due to paresis, because there is an increase in glycogen similar to that found in resting muscles.

The part taken by the brain in the causation of the contractions is rather more obscure and has been somewhat neglected, possibly because consciousness is usually unaffected in the natural disease. The severe convulsive seizures seen more often in the descending type can hardly be explained except by a central origin, since they appear to be occasioned by no obvious stimulus and affect all muscles at the same time. Permin (1914) found that the convulsive attacks could be differentiated from the tonic spasms in the following manner: he produced general tetanus by intravenous injection of toxin while one leg was protected from ascending toxin by intraneural injection of antitoxin into the sciatic. In such an animal the convulsive attacks affected this leg, although no tonic spasms occurred in it; further, when the animal was decerebrated the convulsive seizures ceased, all limbs except the protected leg being, however, still subject to the tonic spasms.

It is thus probable that the general convulsive attacks of tetanus are due to stimuli of central origin affecting the lower motor neurons, while the tonic contractions are due to cord reflexes. There is, however, no reason to suppose that the central stimuli are occasioned by a direct intoxication of the central cells, any more than that the reflex stimuli affecting the cord are due to this factor. The cerebral tetanus produced by Roux and Borrel by direct intracerebral inoculation of toxin appears to have little in common with the general spasms of ascending or descending tetanus.

Histological changes in the nervous system. A number of attempts have been made to disclose the site of the nervous lesion in tetanus by histological means, but without convincing results. The earliest workers found trivial changes in the staining reactions and shape of various cells throughout the nervous system, which later workers ascribed to unsatisfactory technique. Little attention appears to have been paid to this matter since the beginning of the century. Foulerton and Thomson (1900) in rabbits and mice found slight alterations in the straining of the pyramidal cells which they considered specific. Joukowsky (1900) could find no change in guinea-pigs killed at the onset of general tetanus, but in guinea-pigs with chronic tetanus an increase of small round cells was observed about some of the neurons of the anterior horn cells and elsewhere. A partial chromatolysis was also sometimes seen. He regarded the changes in tetanus to be very inconstant and of doubtful significance. Zinno (1903) considered the constant feature to be a gradual disappearance of Nissl's bodies and karyolysis of the neurons and later of the neuroglia. In more chronic cases there was also an infiltration by mononuclear leucocytes. These changes were at first local according to the site of inoculation but later affected many of the cells of the mid-brain, pyramidal cells of the cortex and Purkinje cells. On the other hand, Odier (1904) found fragmentation and solution of the motor end plates and axis cylinders to be constant, as he had found in strychnine poisoning.

The march of the symptoms.

There can be but little doubt that the peculiar progression of the symptoms observed in descending and ascending tetanus is due, as already indicated, to the path of absorption of the toxin. When the inoculation is made intramuscularly the toxin being in immediate relation to the motor nerve endings is taken up by them and causes ascending tetanus. If the dose is large enough the toxin will continue to ascend in the nervous system and affect the cord segments in an orderly sequence one after the other, but this will only be obvious when the dose is so adjusted as to be large enough for this effect, but not large enough to cause intoxication of other peripheral nerves by lymph absorption and blood conveyance. It may be assumed that with even a minute dose lymph and blood absorption takes place, but the amount so absorbed must be relatively large to occasion symptoms (cf. large m.l.d. for intravenous injection).

If blood absorption is entirely prevented by passive immunization of the animal, the purest form of ascending tetanus can be produced (Permin, 1914). On the other hand, when the injection is made under circumstances which preclude any obvious absorption by motor nerves (Zupnik, Sawamura, p. 312) this is effected by the lymph system and the toxin so distributed over the whole body. From the blood the toxin finds its way into the nervous system by a route which is again assumed on reasonable grounds to be the motor nerve endings. This assumption is based largely on the supposition that, if the central nervous system were affected directly from the blood, the whole system should be involved rapidly and simultaneously, and, further, those special forms of tetanus—cerebral and dolorous—should be produced. Actually the onset of symptoms is not general, but again follows a sequence, though less obviously than in ascending tetanus. The curious nature of this sequence is that it tends definitely to start in particular groups of muscles in different species of animals. The reasons for this may be various. It has been suggested that particular nerve groups have a special affinity for toxin, but this seems improbable. Again, if the toxin ascends by the motor nerves, obviously the centres first affected will be those in relation to the shortest nerves. This explanation would seem to be satisfactory to a large extent, but no doubt another factor also has influence, namely, the ease with which an early interference in muscle function can be recognized. Thus a trifling affection of the jaw muscles would be detected before an affection of the face muscles, because the jaw muscles, although not strictly innervated by the shortest nerves, may be detected first in a disorder of function. The facility with which a disorder of these muscles (or other characteristic muscles in other animals) is detected may depend upon the markedly greater power of one set of muscles (the elevators) over the opposing set.

The convulsive attacks, possibly of cerebral origin, which are more obvious in the descending than in the ascending type, are no doubt essentially descending, in that they are due to blood distribution. Permin (1914) has shown that they do not occur in fatal tetanus induced in a passively immunized animal by intramuscular injection—the pure ascending type.

The acceptance of these theories in the case of tetanus in man and the large animals would entail the supposition that absorption of toxin from the local lesion proceeds chiefly by the lymph-vascular route and not by the local motor nerve endings. The possibility of this occurrence has been somewhat overlooked, owing to concentration upon the results of experiments in small animals, but attention has been called to it recently by Albert (1918). If this is so it accounts for the great prevalence of local tetanus during the war; the toxin absorbed by the lymphatics being neutralized in the blood by the prophylactic dose of antitoxin, the results of the smaller nerve absorption become the more obvious. This absorption was seldom sufficient to cause death. It is also noteworthy that in the clinical histories of several of the cases of local tetanus in the literature, it appears that the injuries were such that the toxin would have been produced in close relation to nerve trunks, and so nerve absorption may have been unusually great.

Why lymph absorption should be pronounced in man is open to speculation. It will be understood that in an experimental animal the dose given of toxin or spores is always excessive. A whole lethal dose is inserted at one moment. In man, however, the local concentration of toxin is minute and only produces its effect by continuous elaboration.

It may, therefore, be surmised that these minute aggregations are capable of being removed *in toto* by the lymphatics without nerve endings in muscles being affected.

The incubation period.

According to Meyer and Ransom the incubation period represents the time taken by toxin to traverse the nerve from its periphery to the cell, and, no doubt, this view is in general correct. As shown on p. 309, the smaller the animal, and so the shorter the nerves, the shorter the incubation period, and, also, the further the injection is made from the susceptible nerve cell the longer is this period. The nature of the tissue which receives the injection has, however, an influence also. Absorption is more rapid from muscular tissue, and so the incubation period is shorter. After intravenous injection it is considerably longer ; this must be due to delay in passage of the toxin from the lymph-vascular system to the nerve endings in the muscular system, since the shortest nerves would be available for transport and so the period should be short. It is, of course, understood that these remarks apply to the incubation period following an injection of toxin. In the case of the natural infection there must be added the time required for the germination of the spores.

The General Action of Toxin.

Bullock and Cramer (1919), working with *B. welchii*, indicated that the action of this toxin is to exhaust the suprarenal gland, and that accessory influences such as cold, hyperpyrexia or physical exhaustion, which have a similar action, increase the toxic effect of the culture. It has been alleged by Lesné and Dreyfus (1908) that the effect of tetanus toxin on a guinea-pig can be increased by placing it in the incubator. Marie and Morax (1914) have stated that adrenalectomy in guinea-pigs increases their susceptibility to toxin, but Rogoff and Ecker (1927) have criticized this work and repeated it with an adequate number of animals. They could demonstrate no such effect.

Whether the observations of Vincent (1904) upon hyperpyrexia have any bearing on this matter is unknown. Vincent inoculated guinea-pigs subcutaneously with heated spores and then placed them into an incubator at 40° C. until their temperatures reached 42 to 42·5° C. They were then removed and kept at room temperature. In 2 or 3 days these animals were attacked with a condition which he described as tetanus. They died rapidly, and, after death, tetanus bacilli were grown from the blood and organs. He ascribed this acute 'splanchnic tetanus' to a dissemination of vegetating tetanus bacilli, which was rendered possible by a destruction of the leucocytes by the hyperpyrexia. The alleged frequency of spontaneous tetanus among exhausted troops in hot countries was explained in this way. No confirmation of this work has been published. The Author has repeatedly failed to produce tetanus in guinea-pigs by this procedure. The animals often die 6 or 7 days after the heating but not with symptoms suggestive of tetanus. On the other hand, Ciuca (1907) published a short note without details claiming that the phenomena described by Vincent could be reproduced by subjecting mice for 1 hour to a temperature −2° C.

Natural Resistance to Tetanus.

Considering the wide distribution of tetanus spores and the frequency with which they must gain access to wounds, it is surprising that tetanus is so rare. Reference has been made already to Vaillard's views on the protective action of phagocytosis and to the fact some organisms but not others are capable of activating tetanus spores. Vaillard held that the infrequency of tetanus was due to the rarity of the conjunction of the spore with a suitable activating microbe. A natural immunity to tetanus may clearly result from a mechanism which prevents the germination of the spores or from a mechanism which neutralizes the toxin which is produced after germination. Further, an absolute immunity will exist when the tissues of an animal are not susceptible to the toxin.

Insusceptibility to Toxin.

This phenomenon was studied by Metchnikoff (1897–8). He found that when toxin is injected into scorpions, turtles, alligators, no effect is produced. The toxin circulates in the blood and eventually disappears. As the *species* rises in the animal kingdom there is a tendency to produce antitoxin, but this is not the cause of the lack of symptoms. The tissues are merely indifferent to the toxin. According to Metchnikoff, turtles and alligators are no more susceptible at 30 to 37° C., though the latter produce antitoxin more readily at this temperature. On the other hand, frogs (p. 314) are insusceptible when cold but become susceptible when warmed. Birds are also highly indifferent to toxin, but succumb to very large doses. Sublethal doses merely circulate in the blood and disappear when, after about six days, antitoxin appears. Figures have already been given (p. 308) to show that the susceptibility of other animals is very variable ; the dog, for instance, is 300 times less susceptible than the horse, and it follows that tetanus in the dog must be much rarer than in the horse. McCunn and Fildes (1927) described a case in which the wound contained very large numbers of vegetating tetanus bacilli as compared with the ordinary human case, and this fact no doubt accounted for the intoxication.

When the Wassermann-Takaki phenomenon (p. 339) was first described, it was suggested that the indifference to toxin, or relative indifference, of the animals mentioned above might be due to an inability, or relative inability, of their brain tissues to fix toxin (Knorr, 1897). Metchnikoff (1898), however, found that although the brains of turtles and fowls did not fix toxin neither did a frog's brain, yet this animal was susceptible. It does not, however, seem clear that Metchnikoff took due note of all the experimental possibilities, and this matter might be worthy of further investigation. There has been a suggestion (Birger and Rawitsch-Birger, p. 314) that the immunity of the cold frog is due to an absence of fixation of toxin by the brain at low temperatures. If this is true, it does not explain the immunity of other cold-blooded animals which remain immune when warmed.

Antitoxic Immunity.

Passive. Cattani (1892) showed that a slight degree of immunity might exist in the young of highly immunized rabbits and white rats. This might be due either to transference of antitoxin *in utero* (Ehrlich and Hübener, 1894) or by suckling (Brieger and Ehrlich, 1892). Römer (1908–9) also noted that the calf of a highly immunized cow might have antitoxin in its blood.

Active. Römer (1908–9) suspected that the presence of antitoxin in the blood of bovines was correlated with the existence of tetanus bacilli in their alimentary canals. TenBroeck and Bauer (1923) examined 50 Chinese and found that 26 of these were carriers of tetanus spores in their intestines. All the latter had antitoxin in their blood and so had 2 of the 30 non-carriers. They found that 0·1 c.cm. would neutralize 10 or more m.l.d. (field mice) whereas those called negative failed to neutralize 2 m.l.d. with this quantity. They ascribed the rarity of tetanus among these carriers to the presence of the antitoxin in their blood. On the other hand, Coleman and Meyer (1926) failed to confirm these results during their examination of the sera of 44 persons, members of a population in which the carrier rate was 24·6 per cent. They also used 0·1 c.cm. serum and in no case did this protect against 2 m.l.d. (white mice). TenBroeck and Bauer (1924) also found that 27 per cent. of their stock guinea-pigs were carriers and that the blood of these contained antitoxin. They showed that guinea-pigs without antitoxin in the blood, if fed on spores, would all develop antitoxin in about six months.

In spite of the inability of Coleman and Meyer to confirm this work, there seems to be no reason to doubt it. It is admitted that spores germinate in the gut and presumably produce toxin, and it was shown long ago by Breton and Petit (1908) that toxin introduced into the gut would excite the appearance of antibodies in the blood. It is, however, to be noted that TenBroeck and Bauer (1926) did not claim that this antitoxin occasioned any immunity. They stated that the guinea-pigs with antitoxin in the blood were as susceptible to toxin as the controls. Under these circumstances their previous conclusion as to the effect of antitoxin in the blood upon the incidence of tetanus in man requires reconsideration. It is highly improbable that the amounts of antitoxin which have been recorded in the blood of man can have any serious effect upon the incidence of tetanus. Certainly none has been demonstrated. In diphtheria it is admitted that similar or even smaller quantities are effective, but tetanus toxin is of a much greater potency than diphtheria toxin, and it is well known that very large quantities of antitoxin are necessary to immunize susceptible animals against tetanus.

Phagocytic Immunity.

Tulloch (1919) carried out a number of experiments *in vitro* to show that opsonizing sera of considerable potency might be prepared in rabbits. Best results were obtained by using as antigen a fresh whole culture, but some effect was observed with old cultures, namely with filtered toxin. In the latter case the antigen was presumably present owing to autolysis of the bacteria before filtration. The outstanding feature of these experiments was the demonstration that the opsonic sera were specific to the serological type of bacillus used for the immunization.

In view of the fact that antitoxin had some opsonic activity, it was possible that it had an anti-infective action in addition to an antitoxic. Tulloch found during the war that the incidence of Type I bacilli in cases of tetanus among inoculated men was considerably smaller than among the uninoculated. It looked as if the inoculated had a monotypical immunity against infection. Now commercial antitoxin was found to be made exclusively with Type I strains, and thus would contain some Type I opsonin, and this might account for the results observed.

TenBroeck and Bauer (1926) have described an anti-infective immunity the nature of which they have not yet specified. They found that the guinea-pigs which had been made carriers of tetanus bacilli by feeding, as already mentioned, developed antitoxin and agglutinins in their blood, but they also acquired a resistance to infection which could not be due to antitoxin because it was specific to the type of bacillus fed. Tulloch had already shown that antitoxin prepared against one type was equally operative against all. TenBroeck and Bauer tested the resistance to infection by inoculating them with a small number of spores (1,000) together with aleuronate. This, in controls, caused death in 7 or 8 days. In the test animals the result was as in the controls when the type of spore used was different from that ingested ; when the same, no tetanus was observed. TenBroeck and Bauer also showed that a serum produced by inoculating rabbits with suspensions of bacilli might contain no antitoxin, but would protect against infection monotypically. It might even protect when given 4 days after injection of spores and aleuronate, i.e. 24 hours before symptoms were expected. TenBroeck and Bauer's experiments have not yet been confirmed. They depend, of course, upon a demonstration of the inevitable occurrence of tetanus after an injection of spores and aleuronate. They recognized this, but said that if guinea-pigs are selected which have no gut infection, they will always die after this inoculation. The Author has experienced less certain results. It will be observed that TenBroeck and Bauer's work is an elaboration of Tulloch's. While Tulloch did not claim to have obtained experimentally evidence of protection with his phagocytic sera, TenBroeck and Bauer obtained the experimental evidence but did not associate it with phagocytosis.

In cases where an infection is minimal, and in the presence of an activator which attracts leucocytes without causing any obvious tissue damage, it seems quite probable that an opsonic serum might convert an incomplete phagocytosis into a complete, and thus prevent germination, even though adequate conditions for this were present. On the other hand, the success of the experiment is, in the Author's view, a little precarious, and must require very nicely balanced conditions. On the evidence so far produced, it seems premature to ascribe the rarity of tetanus in man to the fact that he consumes during his life all types of spore, and so is immune to all types. Since TenBroeck and Bauer's conclusion was published, evidence has been provided that tetanus spores are rarely found in the gut of persons in England (p. 322), and, therefore, the immunity of Englishmen at least cannot be associated with a gut infection.

Diagnosis of Tetanus.

Bacteriological methods in the diagnosis of tetanus may be used for the confirmation of a clinical diagnosis or for the detection of infection in cases of accident before the onset of the intoxication. In either case, an attempt is made to detect the tetanus bacillus. Theoretically, according to McClintock and Hutchings's experiments on sheep (p. 337), it should be possible to detect toxin in the blood of a patient 3 or 4 days before the onset of symptoms. So far as is known, however, this technique has not been applied to man. It might possibly be of value in cases of accident likely to develop tetanus, but on the other hand, in such cases it is easier and entails less delay to administer at once a prophylactic dose of antitoxin. The conclusions to be drawn from the discovery of the bacillus are the same as in the case of other micro-organisms; in particular it must be borne in mind that tetanus spores may readily contaminate the skin of the feet of barefooted persons, and that in some populations they are frequently present in the intestinal tract.

It is important when investigating a local lesion to collect as large a quantity of material as circumstances will allow ; at autopsy the whole site should be excised, or if no local lesion is obvious, several suspected sites should be excised and examined. In all such cases the surface of the skin should be cleaned thoroughly, and finally with peroxide of hydrogen. The whole of the available material is then used for an enrichment culture and isolation. The pure culture is tested by agglutination and for toxicity. In cases of urgency, some of the original material after heating may be placed directly into the condensation water of a sloped tube and a diagnosis made, after sufficient practice, on the appearance of the filamentous growing edge after 24 hours. If the number of bacilli in the material is small, however, this method will fail, but positive results are often obtained by subculture of the enrichment culture after 24 hours. In 23 cases said to be cases of tetanus the Author has isolated tetanus bacilli 22 times. The failure occurred with a cotton-wool swab from a wound sent through the post. On the other hand, the difficulty of detecting tetanus bacilli in cases of tetanus has been stressed by many authors. Mackie (1928) was successful in only 2 out of 9 cases.

A note on the bacteria with which *B. tetani* may possibly be confused will be found on p. 365.

As already stated inoculation of animals with original tetanic material or with enrichment cultures usually fails to disclose the presence of tetanus bacilli owing to the destructive action upon the toxin of contaminating bacteria. This method of diagnosis should therefore be discarded.

Immunization.

Tetanus toxin has antigenic properties in the sense that when injected into animals in sublethal doses it readily gives rise to a production of antitoxin which is capable of neutralizing the action of the toxin. For details of the mechanism of toxin-antitoxin reactions, the reader is referred to Vol. VI. In this place are considered the production of antitoxin and the exploitation of the neutralizing action of antitoxin in the treatment and prophylaxis of tetanus.

PRODUCTION OF ANTITOXIN FOR THERAPEUTIC PURPOSES.

BY R. A. O'BRIEN AND A. T. GLENNY.

The production of tetanus antitoxin on the large scale involves the making of large quantities of toxin, the testing thereof, the immunization of horses or mules, and the testing of the antitoxin when obtained. The principles underlying the production of toxin and of immunization of horses are not yet defined with precision ; in consequence, though each laboratory staff can with considerable confidence predict the results it will obtain with its own methods, these methods may be widely different in detail from those used elsewhere. Examples of recent methods pursued in particular laboratories are given in detail by Wadsworth (1927), and by Weinberg and Ginsbourg (1927).

Toxin.

There is no generally used strain comparable to the well-known Park-Williams 8, almost universally employed for making diphtheria toxin. Many strains of the tetanus bacillus will yield good toxin under favourable conditions. The chosen strain may be kept for long periods without detriment. Broth medium inoculated directly from very old stock cultures may give excellent toxin, but the usual practice is to subculture daily for several days from stock cultures into small quantities of the chosen broth medium before inoculating large volumes. There is no evidence that any special composition of medium or technique of anaerobiosis is important. The authors use bottles of 4·5 litres capacity containing 4 litres of medium. The broth is incubated at 37° C. for 5 to 14 days. The reaction in the peptone broth medium with no meat at the bottom of the bottle used by the authors changes slightly after 7 days' incubation from an initial pH 6·5 to between 6·5 and 7·0. The broth is filtered through kieselguhr or porcelain candles. When large volumes are being filtered through candles which allow a satisfactory stream to pass, any slight retention in the candles can be neglected. The clear toxic filtrate may be kept in the liquid form, but since it may rapidly lose toxicity it is wise to precipitate any of it which may be required for testing or observation over long periods. Neutral ammonium sulphate, 700 gms. per litre of broth, gives over-night a thick scum containing most of the toxin present. This is rapidly dried and ground to a fine powder. Sealed *in vacuo* or over P_2O_5 or H_2SO_4 in a desiccator, dried toxin remains stable over long periods. London and Aristovsky (1917) add the sulphate in fractions. For testing purposes, the required amount is weighed rapidly owing to its hygroscopic nature and dissolved in 50 per cent. glycerin; the authors' practice for some years has been to make a strong solution of the dried toxin in 50 per cent. glycerin every two months, and to dilute this as required for each day's testing. The test dose of the strong solution remains constant for at least two months.

For the immunization of horses the toxin is kept in the liquid form, preferably in a cool dark place, either without antiseptic or after the addition of 0·5 per cent. phenol (though probably this antiseptic is harmful to the antigen) or 0·2 per cent. formalin.

Testing Toxin.

The m.l.d. may be found by subcutaneous or intramuscular injection into mice or guinea-pigs. The titres usually quoted range from 0·0001 c.cm. to 0·00005 c.cm. for guinea-pigs weighing 350 gm., and about one-tenth of these quantities for mice of 20 gm., death occurring in 3 to 4 days. The authors have often found that the m.l.d. of a toxin under certain conditions may drop much below its initial value even in 24 hours. For this reason, and because the immunizing value of a toxin rests upon not only its content of ' toxin ' but also on ' toxoid ', it would appear to be safer to use the L+ dose as the measure of immunizing efficiency. This practice has been followed with satisfaction for many years. When mixed with one-fifth of an International Unit of antitoxin, 0·01 c.cm. to 0·1 c.cm. of toxin of an average batch of toxin will kill a mouse of 20 gm. under the conditions specified on p. 357. But even toxin which has lost its toxicity after storage to such an extent that its L+ dose cannot be found has in the authors' experience yielded antitoxin of average titre in horses. By blending such a toxin with antitoxin, one can determine that a union has taken place, i.e. that the toxin has ' binding power ', for if one then adds to the mixture a further quantity of fresh toxin (corresponding to the antitoxin in the mixture), the total mixture is toxic. The deteriorated toxin has bound some of the antitoxin, and so has prevented it neutralizing the toxin added later.

In connection with the ' unit ' of antitoxin, it may be well to point out that the unit here quoted is the International Unit (I.U.) adopted by the League of Nations, and already officially accepted in England and Germany; this equals half the U.S.A. unit, and is, therefore, five times the amount of antitoxin which neutralizes the ' test dose ' of toxin hitherto used when working with the U.S.A. unit. It equals about 1/132 Frankfort Unit. The prophylactic dose of tetanus antitoxin, therefore, which has been usually quoted in the past as 1,500 units (U.S.A.) will now become 3,000 units (I.U.).

Immunization of Horses.

It is not yet clear whether natural immunity exists to any appreciable degree. Buxton and Glenny (1921) tested 500 horses, Ramon and Descombey (1927[2]) 100 horses, without detecting natural antitoxin. The Authors, in a recent examination of this question, have found that though antitoxin is difficult to detect in normal horses, specific antilysin can be found in a certain number, and an occasional horse is found of whose serum 0·00005 c.cm. will prevent lysis of 1 c.cm. of 4·0 per cent. suspension of sheep red cells by tetanolysin. If natural immunity does exist it is probably in the great majority of horses very small, for long experience has shown that when toxin alone is used for immunization, many horses are killed notwithstanding very cautious increase of dosage ; however, even with this method it was noted that a horse would at long intervals be found in serum stables which could tolerate without danger rapidly increased doses of toxin ; such an animal probably possesses considerable natural basal immunity.

Young healthy horses of moderate weight are usually chosen, and injections are given subcutaneously or intra- or intermuscularly. As with most liquid toxins, intravenous injection produces poor immunization. The earliest method was to commence with very small doses of toxin ; this was dangerous. Later, horses were protected with antitoxin given two or three times during the first few weeks of immunization while the doses of toxin were being rapidly increased. More precise control can be gained by commencing with toxin-antitoxin mixtures. Buxton (1921) showed that by giving two doses of slightly under-neutralized toxin-antitoxin mixture at two weeks' interval, horses could be actively immunized and would withstand lethal doses of toxin and culture a month later. The immunization of horses for the production of tetanus antitoxin can, thereafter, be continued with large doses of toxin. Many attempts have been made to produce an atoxic antigen ; iodine has been much tried (Vallée and Bazy, 1917). The modification of toxin by formalin was apparently first described by Salkowski (1898). Formalinized toxin (0·2 per cent. at 37° for several weeks until atoxic) was used by von Eisler and Löwenstein (1912) and Monteiro (1921) (formalin, 0·5 per cent. at 37° C. for 8 days), and has been actively advocated in a long series of researches by Descombey (1924), Ramon (1925–6) and Ramon and Descombey (1927[1]). This valuable method will probably become increasingly used.

Glenny *et al.* (1925) have shown that when immunization is commenced with one or more doses of toxin-antitoxin mixtures, a horse takes about one month to establish a satisfactory basal immunity, and that when a secondary stimulus of toxin is then given the horse responds rapidly by a great production of antitoxin. A group of 10 horses immunized continuously with 2 to 3 doses of toxin a week produced at the end of 6 months antitoxin of average value of 310 I.U., whereas a group of 14 horses given 2 injections and allowed to rest completely for 1, 2 or

3 months gave antitoxin of average value 900 I.U. Basal immunity having been established with mixtures or with toxoid, toxin is given twice or thrice a week in doses increasing by 50 to 100 per cent., and in from 2 to 3 months the average horse will produce antitoxin of about 600 I.U. and upwards per c.cm. Wadsworth (1927, p. 359) gives the detailed record of a horse which in 9 years gave 161 bleedings of about 8 litres, each with an average value of 740 I.U., the maximum titre being 1,400 I.U. It is not often that horses with a titre above 1,600 I.U. are encountered ; the authors have once obtained serum of 2,400 I.U. per c.cm. Schmidt (1928) records 2,940 I.U. per c.cm. By the Banzhaf-Gibson method of 'salting out', antitoxin can be concentrated to about thrice its value ; serum containing 4,400 I.U. per c.cm. was used in the English army during the war for intrathecal injection.

Protection of Horses against Tetanus.

Apart from hyperimmunization of horses for production of tetanus antitoxin of high titre, it is necessary to protect against the risk of accidental tetanus horses used for the production of other sera. A horse being immunized for the production of, e.g. diphtheria antitoxin, may become accidentally infected in the stable with tetanus from some small wound, and blood taken during the incubation period of the attack of tetanus while the horse is apparently in perfect health may contain dangerous amounts of tetanus toxin. For this reason it is necessary that all horses in serum institutes should be immunized against tetanus. The authors have found that a single injection of formalinized toxin to which 2 per cent. potash alum has been added will produce a material active immunity ; a second injection 3 to 4 weeks later considerably increases the antitoxic titre. MacConkey (1926) records the occurrence of accidental tetanus in horses possessing a certain grade of antitoxic immunity after immunization. It is wise, therefore, to give every 6 months an injection of toxoid so that the active immunity is always kept at a high level.

Non-specific Adjuvants.

Walbum (1924) has advocated the use of salts of manganese and other metals for raising the titre of antitoxin in horses. Schmidt (1925) recorded a 10 per cent. increase in the antitoxic titre of a horse being immunized with diphtheria toxin and an increase of from 2½ to 3¼ (Frankfort) units in horses yielding tetanus antitoxin after $MnCl_2$. Pico (1925) could not find any increase in titre of horses being immunized against tetanus though he records increases in other horses. The authors have failed to obtain any significant increase in the titre of horses treated by this method.

Ramon (1926) records that, after observing that horses often gave a higher concentration of diphtheria antitoxin when an accidental abscess appeared, he produced aseptic inflammation with sterile tapioca injected after formalinized toxin ('anatoxin'). When immunizing horses against tetanus Ramon obtained 400 to 500 I.U. per c.cm. (= 10,000 French units) from the horses receiving tapioca, while the control horses gave about

50 I.U. after 4 to 5 months' immunization. It is to be noted that the highest figure reached by the horses receiving tapioca is rather below the average level of horses producing tetanus antitoxin in serum institutes where tapioca is not used. Ramon and Descombey (1927[1]) obtained antitoxin of much higher antitoxic titre from horses given 'anatoxin' when mixed with tapioca or dead streptococci than when given alone. Nattan-Larrier, Ramon and Grasset (1927) gave atoxyl and tryparsamide with 'anatoxin', and record improved antitoxin titres. Glenny *et al.* (1926) have shown that the addition of carefully chosen quantities of alum and turpentine to diphtheria toxin and toxoid increases their immunizing efficiency. These authors have recently found that a single injection into horses of a toxoid containing a small percentage of alum causes a higher antigenic response than toxoid alone.

Type of Bacillus.

The importance of type of bacillus used for immunization was raised by the work of Tulloch (p. 305), but antitoxin made from one strain was found to protect and cure animals injected with any of Tulloch's type strains, and serologists are agreed that there is no evidence demanding the use for the preparation of therapeutic antitoxin of toxin made from all the types.

Antitoxin.

Two methods of dealing with the blood obtained from horses are in vogue; the blood is either allowed to clot naturally and the serum withdrawn, or clotting is prevented with sodium citrate or oxalate, the red cells, which quickly subside, are separated and calcium chloride is added to cause clotting. The resultant serum may be filtered in the usual way through Seitz filters or bacteriological candles. It is immaterial which of these methods of clotting is used.

Sterility and toxicity tests. All serum is tested for sterility on the usual media, preferably after being stored for some months to allow of the growth, and resultant ready detection, of any of the rare 'phenol-resisting' organisms which may be accidentally introduced during preparation. It is tested for toxicity, 5 c.cm. being injected subcutaneously or intraperitoneally into guinea-pigs and possibly 0·5 c.cm. into mice.

Testing of antitoxin. Serum is tested in the usual way by mixing the test dose of toxin with various fractions of a c.cm. of serum, and after allowing the mixture to stand at room temperature for not less than an hour, injecting subcutaneously into a guinea-pig of approximately 350 gm. weight, or into mice of 20 gm. weight. The quantity of serum which protects a guinea-pig for 4 days contains 0·2 I.U. (vide p. 354). Injection into mice is made at the base of the tail.

It has been suggested (Madsen, 1923) that the more convenient measure may be the Lo level, i.e. the amount of antitoxin which just prevents the appearance of any symptoms. Such titrations are theoretically sounder than L+ determinations, because they measure more nearly the point of true neutrality. The Lo method of testing, moreover, makes a double appeal to the serologist; one may kill animals directly definite symptoms of tetanus are seen, without waiting for them to die in order to indicate the L+ level; half the animals survive without symptoms, thus considerable economy is affected. The Lo determination in tetanus is of greater practical importance than in diphtheria, where the end point of local œdema is more difficult to read than such easily recognized symptoms as tetanic rigidity.

Madsen has further shown that the results of comparative titrations of sera may differ according to the endpoint adopted. The Authors suggest that the difference may be due to differences in 'avidity' between sera otherwise apparently identical; this point is at present under observation.

Flocculation. Abt and Erber (1926) have used flocculation as a means of titration. It appears, however, to be difficult to get completely uniform results. Weinberg and Ginsbourg (1927), remark that this method of titration succeeds 'in the majority of cases'. Schmidt (1928) is of opinion that the flocculation does not always give the 'neutral point', and that it is always necessary to use also an animal test. In the Authors' experience many sera fail to flocculate and suggestions of 'double zones' and even occasional 'treble zones' of flocculation have been met with. It is possible that many factors take part in the Ramon tetanus flocculation, e.g. an interaction between toxin and antitoxin, bacterial protein in the toxin which is dissolved in the broth during the growth of the culture, and a precipitating antibody in the antitoxic serum, as well as hæmolysin in the toxin and anti-hæmolysin in the antitoxic serum. The influence of these different factors has not yet been clearly defined. Some part of the flocculation phenomenon may be due to other substances than the specific antigenic toxin, but this latter takes definite part in the flocculation, for the Authors have found that the precipitate separated and washed after flocculation will immunize guinea-pigs against tetanus.

R. A. O'B. and A. T. G.

TREATMENT OF TETANUS BY ANTITOXIN.

The first case of tetanus in man—a case of trismus neonatorum—was treated by Baginsky on January 30th, 1891, with 1·5 c.cm. of a rabbit serum prepared by Kitasato. This patient did not recover (Baginsky, 1891). In Italy the first case to be published (Schwarz, 1891) was treated on September 18th with Tizzoni's dry dog antitoxin. This patient recovered, as indeed did most of the early Italian cases, but it seems probable that these were selected, since the incubation periods when reported were long. The first cases in France were treated by Roux and Vaillard (Rénon, 1892) on April 2nd and 13th, 1892, with Vaillard's defibrinated rabbit's blood antitoxin. Both cases died. In England the first case reported (Winfield-Roll, 1894) was treated with Roux's dry antitoxin on October 27th, 1893. This was described as the first successful instance of the use of this form of treatment.

The treatment of tetanus with antitoxin did not result in the favourable outcome which had been anticipated and has not done so to this day. Nevertheless there is no doubt that this treatment is not so ineffective as individual authors have concluded on insufficient experience. Taking into consideration only large unselected series, it is clear that the mortality in treated cases is lower than in pre-antitoxin days and also than in post-antitoxin days in the absence of this treatment. For instance, it has been shown by Permin (1914) that in pre-serum days the mortality was 79 per cent., but in 189 cases treated with serum it was 57·7 per cent. At the London Hospital the mortality in untreated cases since 1878 has been 88·7 per cent., while in 103 treated cases it has been 71·8 per cent. These results are not striking, but, nevertheless, indicate a certain therapeutic advantage in the use of antitoxin. As shown in a subsequent section the lack of success is often attributed to a lack of penetration of the antitoxin into the central nervous system. The intrathecal route is, therefore, generally recommended and adopted. Nicoll (1915) treated 20 consecutive and unselected cases in New York with remarkable success. He gave 3,000 to 5,000 units intrathecally and 10,000 units at the same time intravenously; he repeated the intrathecal injection in 24 hours and gave 10,000 units subcutaneously 3 or 4 days later. There were only 4 deaths in this series. Results comparable with these have not, however, been reported subsequently. In Dean's (1917) 14 cases treated intravenously with 30,000 units there was only 1 death, but it should be noted that all had probably been treated prophylactically and that the incubation periods were long.

The incubation period has the same prognostic importance in treated cases as in untreated, and the outlook is always better when the march of the symptoms is slow.

Treatment of Experimental Tetanus by Antitoxin.

The difficulty of influencing tetanus in experimental animals with antitoxin after the appearance of symptoms was recognized early. Kitasato (1891) infected 12 mice with splinters steeped in a heated spore suspension. In 48 hours definite symptoms were present and 10 were given 1 c.cm. antitoxin intraperitoneally. The 2 untreated controls died in 55 hours. On the following day a further dose (1 c.cm.) was given, but 5 died in 80 hours. The remaining 5 eventually recovered after a further dose of antitoxin. Similarly, with guinea-pigs no less than 27 c.cm. in 4 doses was necessary to cure. Equal inefficiency has been noted by subsequent workers and indeed it is frequently stated that the effect of antitoxin is inappreciable. This, however, is undoubtedly not the case. Little attention need be paid to the results of subcutaneous and intramuscular injections, since absorption in such cases is slow and there is no doubt that the time factor in treatment is all important. Further, the dosage has often been small. If, however, larger doses are given by the intravenous route and not too late in the disease, recovery is almost constant.

For instance, guinea-pigs inoculated with 4 m.l.d. toxin or with earth and spores may be saved by intravenous injection of a dose equivalent to half a unit per gm. weight given about 24 hours after the onset of local tetanus. If, however, treatment is delayed beyond this point, recovery is infrequent. This relative inefficiency of treatment has led to speculation as to its cause.

Lack of Penetration of Antitoxin to the Central Nervous System.

Blumenthal (1896) noted that the blood of a case of tetanus after antitoxin treatment contained no toxin but that the cord of the same case was toxic. From this he deduced (1897) that the toxin in the nervous system was so firmly bound to the nerve structures that it could not be neutralized by antitoxin. Dönitz (1897) found that if antitoxin is delayed in experimental tetanus, the animal can be saved up to a certain point by increasing the dose in proportion to the delay. He concluded that the fixation of toxin can be broken down by a sufficiently large dose of antitoxin.

This hypothetical fixation of toxin by nerve tissues was established by Wassermann and Takaki in the following year, or rather they published experiments which were shown by others to illustrate this effect (p. 339). The phenomenon of fixation of toxin by nerve tissue is generally accepted as being one of the causes of the incurability of tetanus, the toxin being fixed to such a degree that antitoxin can scarcely remove it from combination. On the other hand it must be remembered that the fixation demonstrated by Wassermann and Takaki is purely an *in vitro* phenomenon and that there is no direct evidence that it occurs *in vivo*. Furthermore, if *in vitro* experiments have any application, the evidence is in favour of a relatively easy dissociation of the toxin-nerve complex by antitoxin (Besredka, 1903; Noon, 1907).

A more obvious explanation for the relative incurability of tetanus might be found in the fact that the central nervous system is outside the influence of many substances introduced into the blood-lymphatic system. It has been noted already that toxin injected intravenously cannot penetrate directly into the central nervous system, because if it did cerebral tetanus would follow. It is also well known that many diverse substances injected into the blood are unable to penetrate into the central nervous system; for instance, salvarsan (McIntosh and Fildes, 1914), certain aniline dyes (McIntosh and Fildes, 1916) and horse serum (Teale and Embleton, 1919–20).

Little information appears to be available upon the distribution of antitoxin after injection. Ransom (1900[1]) showed that after intravenous injection it remained at a higher concentration in the blood than in the lymph, although it passed into this in a few minutes. Toxin in the blood is neutralized in 15 minutes (Ransom, 1900[2]). When injected subcutaneously it is carried to the blood by the lymph stream. These effects are the same whether horse or dog antitoxin is used in the dog. Concentration in the blood is slow after subcutaneous injections (Dehne and Hamburger, 1907).

So far as concerns the penetration of antitoxin into the central nervous system, no information is available, except in the case of the cerebrospinal fluid. But penetration into this fluid cannot be accepted as evidence of penetration into the brain or cord. Mere traces only are found in the cerebrospinal fluid even after large intravenous doses (Ransom, 1900–1). On the other hand, Dean (1917) found that 12 hours after injection of 30,000 units intravenously in man, 1 c.cm. of the cerebrospinal fluid neutralized 100 m.l.d., but in 4 other cases examined later (19 hours to 6 days) the amount of antitoxin found was trivial. Conceivably the passage of antitoxin to the fluid may depend upon an urticarial alteration in the permeability of the meninges.

Whatever the reason, it is certain that a high concentration of antitoxin in the blood has little or no influence on toxin introduced into the nervous system. Roux and Borrel (1898) and Marie (1907) produced cerebral tetanus by direct inoculation into the brain of an animal which had been highly immunized, and had a high antitoxin titre in the blood. The experiment may fail if any slight hæmorrhage occurs, owing to neutralization of the inoculum. Meyer and Ransom (1903) found that injections of toxin into the sciatic were uninfluenced by a high degree of active or passive immunity. The same effect is illustrated by the production of local tetanus in an immunized animal even after intramuscular injection (Permin, 1914), or the natural development of local tetanus in men after prophylactic injections of antitoxin. In such cases it is presumed that the local concentration of antitoxin is insufficient to neutralize all the toxin introduced, and that some is able to be absorbed by the nerves, when it produces its effect without hindrance.

Attempts to introduce Antitoxin into the Central Nervous System.

The recognition of the inefficiency of blood-borne antitoxin led Roux and Borrel (1898) to attempt a direct introduction of antitoxin to the central nervous system. Their method was to inoculate the serum intracerebrally, and they reported that guinea-pigs which already showed stiffness of the hind limb could be saved by intracerebral injection of a quantity of antitoxin, multiples of which were quite ineffective intravenously or subcutaneously. The fate of antitoxin injected intracerebrally was followed by Ransom (1901). It passes rapidly into the cerebrospinal fluid and blood, so that practically the whole may be accounted for in the blood after 24 hours. The same result, of course, follows intrathecal inoculations. The impracticability of inoculations into the cerebrum, cord or nerves for the treatment of tetanus, and the ease with which drugs can be introduced into the cerebrospinal fluid has naturally led to the exploitation of this route. There is, however, little reason to suppose that substances injected into this fluid are in an optimum position for influencing toxin in the tissue spaces of the cord. The anatomical and physiological relations of the cerebrospinal fluid are still obscure, but the recent investigations of Weed (1922) indicate that so far as the substance of the brain and cord is concerned it receives small aggregations by an *outward* flow along the perivascular channels. The flow of cerebrospinal fluid is, therefore, not directed to the areas which are assumed to be the site of intoxication. Nevertheless, there is some evidence that intrathecal injections are more effective than injections by other routes (Sherrington, 1917). Monkeys were inoculated into the gastrocnemius muscle with 8 m.l.d. toxin, and when local signs were developed, 1 dose of antitoxin consisting of 2,000 units per kgm. monkey was administered.

The following results were obtained :

25	monkeys	subcutaneously	2	recoveries.
25	,,	intramuscularly	3	,,
25	,,	intravenously	7	,,
25	,,	intrathecally lumbar	14	,,
20	,,	,, bulbar	13	,,

Park and Nicoll (1914) treated 350 gm. guinea-pigs by different routes 17 to 24 hours after injection of 2 m.l.d. toxin intramuscularly.

The following results were obtained :

250	units, intravenously	3	out of	5	died.
200	,, ,,	2	,,	3	,,
100	,, ,,	5	,,	5	,,
250	,, , intrathecally	2	,,	8	,,
50	,, ,,	1	,,	4	,,
10	,, ,,	0	,,	4	,,

Florey and Fildes (1927) pointed out that this result was surprising, since a diminution in the dose of serum intrathecally actually led to more cures. No doubt the numbers were too small for just conclusions. These authors were unable to satisfy themselves that intrathecal injections in guinea-pigs by the technique of Park and Nicoll were reliable. Others have failed to detect any difference in the number of cures obtained by intrathecal injection. Florey and Fildes (1927) sought to overcome the difficulty that the cerebrospinal fluid is an excretion by utilizing the procedure of Weed by which the flow of this fluid can be reversed. Fifteen c.cm. of 25 per cent. NaCl were injected intravenously into rabbits followed by 1,000 U.S.A. units intrathecally, and, although there was evidence that the antitoxin flowed into the brain, no advantage could be detected in this method.

Other workers have tried to alter the permeability of the cerebral capillaries to allow direct passage of the large molecular antitoxin from the blood. Trabaud and Baur (1924), for instance, ascribe some of the good effect of chloral and morphia to this cause. Le Fèvre de Arric and Millet (1927[1]) found that urotropine led to a slight passage of antibodies into the cerebrospinal fluid. In the case of antitoxin (1927[2]), when this was given with urotropine intravenously before an injection of toxin into the brain, the animal either recovered or survived longer than the control. Urotropine, however, increases the permeability of the capillaries or cells to toxin, so that if urotropine and toxin are given together, an ineffective dose becomes fatal (1926). For treatment, therefore, urotropine is likely to be dangerous. The Author has found this to be the case, urotropine and antitoxin failing to cure when antitoxin alone succeeded.

Finally, Speransky (1927) states that if an animal's theca is punctured and the cerebrospinal fluid pumped backwards and forwards, remarkable therapeutic action can be obtained in some diseases from serum introduced into the blood stream. The method, however, in tetanus was a failure, at any rate no great claims were made in this disease.

The general conclusion to be drawn from these experiments seems to be that if there is any advantage in the intrathecal route for introduction of antitoxin, it is unimportant in the cure of experimental tetanus. The failure to cure tetanus depends upon factors other than the site of injection of antitoxin.

PROPHYLAXIS OF TETANUS BY ANTITOXIN.

Antitoxin was first used prophylactically on a large scale by Nocard (1897) in an attempt to diminish the high mortality from tetanus in animals after operation. He distributed serum to 63 veterinary surgeons. Among 2,300 animals which received 2 doses shortly after an operation, no cases of tetanus occurred, while only 1 case (with recovery) was seen when the treatment was given up to 4 days after an accident. During the period of observation, however, the 63 veterinarians dealt with 259 cases of tetanus in untreated animals. The results are surprisingly good, but there appears to be no recent publication by which to check them.

At the outbreak of the war, however, it soon became obvious that tetanus was to exert an influence which was lacking in recent campaigns, and measures had to be taken to provide in December, 1914, 200 times more doses than were available in August. Among all belligerent nations it became the endeavour to inject every wounded man with a prophylactic dose of antitoxin. The dose adopted in the British Army was 500 U.S.A. units, and this was considered adequate (MacConkey and Homer, 1917), but in civil practice at least 1,500 units are usually given. The general effect of this action is shown by statistics relating to wounded who had been returned to hospitals in England (Bruce, 1920–1).

		Ratio of tetanus to wounded	*Injected or not*	*No. of doses of antitoxin sent to France. (Cummins, 1923)*
1914	Aug.	3·7 per 1,000 wounded	Partially	600
	Sept.	9·0 ,, ,,		13,950
	Oct.	7·3 ,, ,,		84,880
	Nov.	2·3 ,, ,,	Mostly	106,000
	Dec.	1·4 ,, ,,		130,000
1915	Jan.	0 ,, ,,		
	Feb.	1·1 ,, ,,		
	Mar.	0·4 ,, ,,		
	Apr.	2·3 ,, ,,		
	May	0·8 ,, ,,		

Effect of prophylaxis on mortality.

Protected	Mortality 22 per cent.
Not stated and unprotected..	,, 53 ,,

Effect of prophylaxis on mortality from tetanus in ' trench feet '.

Protected	Mortality 16 per cent.
Unprotected	,, 75 ,,

It will be apparent from these figures that tetanus was not abolished by this treatment, neither was the mortality abolished in such cases that occurred. The prophylactic treatment of tetanus does not, therefore, afford such an absolute freedom from the disease as is found in the case of diphtheria. The explanation of the failure is to be found in three main considerations.

(1) When 500 units are administered the actual titre in the blood is never high, and if the person receives an extensive injury infected with tetanus bacilli or a smaller injury infected with highly toxic tetanus bacilli, the amount of toxin produced can easily swamp the antitoxin in the blood. In tetanus the toxin is of a much greater potency than in diphtheria.

(2) The titre of antitoxin in the blood falls rapidly after the injection and is often small within 10 to 14 days. If at this time the wound is still in such a condition that spores contained in it can germinate and produce toxin, tetanus will result. This fact accounts for the prolongation of the incubation period observed in immunized soldiers.

Incubation in protected soldiers = 45 days.
,, in unprotected ,, = 11 ,,

It also illustrates the necessity for surgical treatment of the wound to prevent germination of spores after the effect of antitoxin has diminished.

(3) Even where there is a high concentration of antitoxin in the blood as in immunized animals, the quantity in any one place will be insufficient to deal with a considerable production of toxin. In such a case it will always be possible for some of the toxin to be absorbed by the local motor nerve endings.

This phenomenon accounts for the great frequency of local tetanus during the war.

1914 ..	1	local per 100 cases of tetanus.
1915 ..	1	,, ,, ,,
1916 ..	13	,, ,, ,,
1917 ..	23	,, ,, ,,
1918 ..	16	,, ,, ,,

The ' descending ' signs are prevented by the antitoxin in the blood stream, leaving the pure ' ascending ' type of the small experimental animal.

The enormous potency of tetanus toxin and the double route of absorption makes it obvious that antitoxin can never supply the same degree of security from tetanus that diphtheria antitoxin does from diphtheria. This, however, is not to be taken as a condemnation of tetanus antitoxin, since the security from diphtheria is almost absolute. It, however, indicates that judgment as to what a ' safe ' prophylactic dose of antitoxin is—either in the case of animals or wounded soldiers in a campaign—depends upon considerations of expediency rather than experiment. No dose can be looked upon as giving security from local tetanus under the conditions of some wars.

Bacteria which resemble B. tetani.

A number of bacilli exist which are capable of being confused with *B. tetani* because of the presence of typical drumsticks in cultures, either as isolated examples among many without this appearance or as the predominant feature. There is considerable difficulty in dealing with these bacteria with confidence because the named varieties have been studied from relatively few samples, and the characters described are not exclusive, but represent mere differences in degree of characters common to all. In routine work with this group, also, bacteria are commonly met with which cannot be clearly identified with the type strains of any known variety. As Fildes (1927[1]) pointed out, if this group is looked at as a whole it appears as a gradation of bacteria whose chief link is that they form with more or less facility a round terminal spore. At one end of the series fermentation of sugar is a conspicuous feature, and at the other, digestion of protein is well marked. In the centre come a number of strains which appear to have little or no action on these substances, while *B. tetani*, the only pathogenic variety, appears early on the proteolytic side.

From the practical point of view, the matter of importance is that when these bacteria are grown upon a digest medium such as Tulloch's, they exhibit a marked tendency to develop drumstick spores which are indistinguishable from those of the ideal tetanus bacillus. When, however, they are grown upon an ordinary laboratory medium, those on the proteolytic side fail to develop spores at all, or if they do, they are more obviously oval or very large or attached to long filaments. Such samples differ obviously from an ideal tetanus bacillus, but they do not differ obviously from tetanus bacilli which are themselves aberrant. They cannot, therefore, be distinguished absolutely by their morphology from tetanus bacilli.

It is also important to remember that these non-saccharolytic varieties preferably grow in a film on the surface of the medium, as does *B. tetani.* They are difficult to place with certainty within the limits of any named variety. The Author would be inclined to look upon them as non-putrefying strains of *B. putrificus* (vide *infra*) were not this identification incongruous. The only strain of *B. putrificus* which he has examined has no tendency to grow as a film, but this may be merely a ' rough ' change whereas the Author's technique of isolation favours the collection of ' smooth ' strains.

Certain named varieties deserve brief mention. In some cases they may be synonymous with varieties insufficiently described by older workers.

B. putrificus Bienstock, emend. Sturges and Rettger, 1919. This is the organism which, according to Sturges and Rettger, is the same as the species named *B. putrificus* by Bienstock (1884). At the present day, it is, of course, impossible to assert that the strains issued by Sturges and Rettger (National Collection of Type Cultures, No. 2221) as *B. putrificus* are the same as those studied by Bienstock in impure culture, but, nevertheless, confusion may be avoided by retaining Bienstock's species name for his bacillus as emended by the later authors. Knowledge of this organism is based upon the work of Reddish and Rettger (1922–3–4) who definitely established its existence in opposition to the opinion of the Medical Research Committee (1919) that it represented a mixture. In morphology when grown on an ordinary medium the bacilli are often long and filamentous. Spores are few, but usually large and round, situated at the end of a filament. They are late in appearance. In Tulloch's medium, however, they resemble *B. tetani* quite closely. In culture no film is found. The colonies are said to be clear and transparent, but in the strain examined (N.C.T.C.), they were opalescent and speckled, afterwards more opaque. No carbohydrates were fermented. Gelatin was liquefied and in Robertson's medium after an interval of seven days a progressive digestion was observed. This latter is looked upon as the outstanding feature of the organism. It is more active and rapid in Tulloch's medium than in Robertson's, and is increased by repeated heavy subcultures.

B. cochlearius Douglas, Fleming and Colebrook, 1920, appears to the Author to be identical with *B. putrificus*, except that the degree of digestion of meat was not so pronounced as with *B. putrificus* in the one specimen examined. This should be discarded as a separate species.

The numerous unnamed strains mentioned above may also be looked upon as belonging to this group, but the digestion of meat is still less pronounced.

B. tetanomorphus Medical Research Committee, 1919. This name was applied by the Medical Research Committee (1919) to the 'Tetanus-like bacillus—Type No. IX', described by McIntosh (1917). Morphologically it resembles *B. tetani*; even on undigested media spores are abundant. Colonies are readily formed on the surface of a medium. They are usually flat and of little opacity. Meat medium is not digested and gelatin not liquefied. Glucose and maltose are fermented. Adamson and Cutler (1917) described a very similar bacillus. The colonies are, however, described as being moist and raised; further, lactose is fermented in addition to glucose and maltose. American authors refer to a '*B. tetanoides*', the nature of which is not certain, but which does not appear to differ conspicuously from *B. tetanomorphus*.

B. sphenoides Douglas, Fleming and Colebrook, 1920. The resemblance of this bacillus to *B. tetani* is less marked. The bacillus is short and fusiform and often in pairs end to end. Spores are formed readily; they are large and round, and when mature, terminal. The remnant of the body is wedge shaped and this becoming thinner the appearance resembles somewhat *B. tetani*. Surface colonies are small and transparent. No digestion of meat or gelatin is observed. Glucose, maltose, lactose and salicin are fermented, sometimes also mannitol and saccharose.

B. tertius Henry, 1916. This is a slender curved bacillus which forms freely large terminal oval spores. Isolated examples are, however, very like *B. tetani*, especially in Tulloch's medium. Colonies are small and transparent or opalescent. No digestion of meat or gelatin is seen, but the following sugars are fermented: glucose, maltose, lævulose, saccharose, lactose, mannitol and salicin.

Some of the characteristic details of these organisms are the subject of dispute.

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CHAPTER XI. *BACILLUS BOTULINUS.*

BY R. TANNER HEWLETT (SEAMEN'S HOSPITAL, GREENWICH).

WITH SECTIONS BY

W. BULLOCH AND R. A. O'BRIEN.

THE name *Bacillus botulinus* may conveniently be applied to a group of related organisms which are the cause of a form of food-poisoning in man known as ' Botulism '.

Botulism results from the ingestion of the preformed toxin of, and not from infection with, these organisms, the toxins of which, while not strictly identical, all possess very similar physiological activities.

History.

BY W. BULLOCH.

Attention was first directed to sausage-meat poisoning in 1820 by the mystic poet-physician, Justinus Kerner, in Würtemberg, and by Paulus (1834). Full accounts of the early literature will be found in the extensive communication of Müller (1869, 1870), but it was not until 1897 that our modern knowledge may be said to have begun by the discovery of van Ermengem of Ghent, that the cause of this form of food poisoning is an anaerobic organism—*Bacillus botulinus.* In December, 1895, a typical outbreak of botulism occurred among the members of a musical society who were called to perform a dirge at a funeral in the village of Ellezelles (Hainault). After the ceremony they were regaled with a cold collation in which a salted ham played a dominant part. The ham had been taken from a healthy pig slaughtered in August, 1895, and in the fresh state had been partly consumed with impunity. The remainder which had lain in brine for four months, however, was incriminated as the cause of the outbreak. It affected 34 persons, of whom 3 died. The symptoms came on 24 to 36 hours after the consumption of the ham, and involved, in particular, certain cranial nerves, as was shown by the presence of squinting, diplopia, ptosis, dysphagia and aphonia. In the spleen of one of the fatal cases, and also in the salted ham, van Ermengem found a sporing anaerobe, which he isolated and studied with great exactness, and by means of the living cultures, as also of the soluble filtrate from these cultures, he was able to produce in animals all the clinical phenomena which had been seen in the affected persons. By means of van Ermengem's cultures, W. Kempner (1897) in Berlin showed that the botulinus toxin causes the development of a powerful specific botulinus antitoxin in the body of goats. Confirmation of van Ermengem's observations was made by P. Römer (1900) in a small epidemic in Upper Hesse, and by Landmann (1904) in Darmstadt in an epidemic following the consumption of tinned beans. Knowledge of outbreaks of botulism grew, however, very slowly until reports began to arrive from the United States about 1915 of a wide dissemination of the disease (Dickson, 1915, 1918), and since this time a great deal has been written, not only on the disease itself, but also on the life history and characters of *B. botulinus*. Much of this new knowledge we owe to the laborious work of K. F. Meyer and his co-workers in California from 1921 onwards.

W. B.

Epidemiology.

Bacillus botulinus is an elusive organism, difficult to isolate without a special technique. Until 1913 it had been isolated, or its toxin identified, only in a few European outbreaks other than the Ellezelles one, and the natural habitat of the organism remained unknown. The foodstuffs concerned in these outbreaks of botulism were ham (four instances), salt fish (once), blood sausage (once), and preserved beans (once). The last-named outbreak occurred in Darmstadt in 1904, and is of interest as being the only occasion until then on which a vegetable foodstuff had been implicated, and because the strain of *B. botulinus* isolated therefrom by Landmann (1904) proved to be different from the Ellezelles strain isolated by van Ermengem.

Apart from three or four doubtful cases, only a single outbreak of botulism has occurred in the British Isles, that at Loch Maree, Ross-shire, in 1922 (Leighton, 1923). The material causing this outbreak was potted wild-duck paste. Eight persons were attacked, all of whom died.

Until 1909, botulism had not been definitely recognized in the United States, but since then the disease has been relatively prevalent, and it has been estimated that up to 1926, 146 outbreaks had occurred in the United States and Canada, with some 500 cases and 330 deaths, a mortality of 66 per cent.

Records have been collected by Bitter (1921) of 68 reputed outbreaks in Germany between 1898 and 1919, with 298 cases and 48 deaths.

Some obscure diseases of animals and poultry have, during the last few years, been shown to be manifestations of botulism (see p. 383).

Bacillus botulinus.

'TYPES' AND 'SPECIES' OF BACILLUS BOTULINUS.

The description of *Bacillus botulinus* as originally given by van Ermengem must be considered to be the authentic description of the type species of the organism. This was the strain isolated in the Ellezelles outbreak of 1895, and may be referred to as the 'Ellezelles strain'. Other strains isolated in later outbreaks of botulism occurring in Europe were observed to differ in some of their cultural and other characters from the Ellezelles strain. Thus the Darmstadt strain of Gaffky and

Landmann (1904), isolated in an outbreak due to the consumption of preserved beans, was not quite identical with the Ellezelles strain. A similar observation was made by van Ermengem himself in another outbreak at Iseghem. Lastly, Leuchs (1910), in a study of the toxin and antitoxin relationships of strains of *B. botulinus*, showed that the Ellezelles and Darmstadt strains were distinct, inasmuch as the antitoxin derived from the toxin of the one did not neutralize the toxin of the other, although the toxin of each organism was completely neutralized by its corresponding antitoxin. Leuchs also observed that the Darmstadt differed from the Ellezelles strain in the following respects: the rods were more slender and less Gram-positive, the flagellation was not quite the same, and gas was very scanty or absent in glucose agar. The Ellezelles and Darmstadt strains are probably now lost, so that it is somewhat problematical to which of the modern ' types ' they belonged.

Among the strains of *B. botulinus* which have more recently been isolated in America and elsewhere, similar immunological and other differences have been observed, and the terms ' type A ' and ' type B ' were introduced by Georgiana Burke (1919²) to distinguish two groups of them. Burke studied 12 strains of *B. botulinus* by means of toxin-antitoxin neutralization tests and found that they formed two groups, the antitoxin of one group neutralizing the toxin of its own group, but not that of the other or heterologous group. To these two groups the designations ' type A ' and ' type B ' were given. Type A strains were mostly derived from outbreaks of human botulism that had occurred in California, and type B strains from cases of forage poisoning in animals, together with one strain isolated by Nevin (see 1921) from cases of human poisoning caused by cheese. Type A strains usually produced a stronger toxin than type B strains, but their general morphological and cultural characters were almost identical. Later work has, however, shown that the members of a particular type-group may exhibit some cultural and other differences. Dickson had previously observed that the Nevin cheese strain differed immunologically from other strains with which he was working, and he and Howitt (1920) afterwards confirmed Burke's work.

A third group was afterwards identified by Bengtson (1922), and following Burke's nomenclature, was designated ' type C '. It was isolated from the larvæ of *Lucilia cæsar*, one of the green-bottle flies, reputed to have been concerned in a ' limberneck ' epizootic of chickens. An active toxin is formed by it, which, however, is not neutralized by considerable amounts of types A and B antitoxins.

Finally, there is Seddon's *Bacillus parabotulinus*, causative of a paralytic disease of cattle, which has considerable resemblance to Bengtson's type C, and the toxin of which is neutralized by type C antitoxin, though its own antitoxin does not neutralize type C toxin.

Bengtson has also used the term ' *Clostridium parabotulinum* ' to designate certain races of the type A and type B strains, and these must not be confused with Seddon's *B. parabotulinus* (see p. 384).

MORPHOLOGICAL AND CULTURAL CHARACTERS OF BACILLUS BOTULINUS.

Van Ermengem's Observations.

Van Ermengem (1897[2]) made a careful and complete study of the Ellezelles strain of *Bacillus botulinus*, and there can be no reason to doubt the accuracy of his description of this particular strain, though the characters he ascribed to it may differ to some extent from those of more recently isolated strains. The following is a summary of his description :

Morphology, &c. A large straight rod with rounded ends, measuring 4μ to 6μ in length, by 0·9μ to 1·2μ in breadth. It occurred singly and in pairs, rarely in short filaments. The organism was feebly motile and possessed from four to eight fine flagella. It was Gram-positive, though not markedly so. Spores were formed, generally terminal, ovoid, and somewhat wider than the rod; clostridial and spindle forms sometimes occurred.

Cultivation. The organism was a strict anaerobe and developed best between 20° C. and 30° C. ; at 37° C. growth was poor, spores were not formed, and very little toxin was produced.

The colonies in a glucose gelatin plate were at first (4 to 6 days) circular and transparent, yellowish-brown in colour, and contained refringent granules in movement, especially at the periphery. After many days, club-shaped rays emerged from the colony, giving the edge a woolly appearance. Gelatin was slowly liquefied, but milk was neither changed in reaction nor curdled. Abundant gas was formed in glucose agar but there was no fermentation of lactose and saccharose. The cultures had a rancid odour, due to butyric acid. A slightly alkaline (to litmus) medium was most favourable for growth. The vitality of the spores was destroyed in half an hour at 80° C.

Toxin was formed in culture, and was also obtained from the ham, the cause of the outbreak, which was very toxic to many animals by inoculation and, in larger doses, by ingestion.

Cultivation and Cultural Characters of Bacillus botulinus.

It will now be convenient to survey at greater length the methods of cultivation and cultural and other characters of *B. botulinus*, which emerge from the more recent work upon the organism, and at the same time to describe more fully some of van Ermengem's observations.

B. botulinus (*Clostridium botulinum* in American nomenclature) is a toxicogenic organism widely distributed in nature. It is an inhabitant of the soil, and by contamination with, or derivation from, the soil, foodstuffs of man and animals become infected with it, and if conditions be appropriate the toxin is formed.

Cultivation. B. botulinus, while not particularly fastidious in its nutritive requirements, grows much better in some media than in others, and for maximum toxin production special media have been devised. It will grow in nutrient agar, better in glucose- and in blood-agar, in nutrient broth and glucose broth, in milk, gelatin, and peptone-water. A broth containing minced meat, such as Robertson's (vide Vol. IX), is to be preferred, and a reaction of pH 7·4 is most suitable. For isolation, a liver agar, or a special meat-mash liver-digest broth (see Vol. IX), is to be preferred, and the last named is very suitable for toxin production.

Whereas van Ermengem found a temperature of about 30° C. best for growth and toxin production, the optimum temperature for American strains is 37° C.

Failure of growth in cultures, which is not infrequent, may depend in part upon the degree of seeding and of anaerobiosis. Plentiful seeding is more likely to be successful than scanty seeding, and Rockwell (1924) maintains that the growth of some anaerobes, including *B. botulinus*, is delayed or prevented in the absence of a small quantity of carbon dioxide, if the medium be thinly seeded. Dack *et al.* (1927) found that types A and B grow well in a veal broth at 37° C. when the air is replaced by carbon dioxide up to a pressure as high as 500 mm. of mercury. With the air replaced by oxygen there was no growth at a pressure of 50 mm. of mercury, and in a partial vacuum growth occurred when the air pressure was 40 mm. or less, but not when it was 80 mm. or more. *B. botulinus* is not, therefore, a stringent anaerobe, and like other anaerobes will develop aerobically in association with certain aerobic bacteria (Francillon, 1925), as first noted by van Ermengem.

Colonies. In nutrient agar with 2 per cent. of glucose, the colonies in an anaerobic plate, or in a shake culture, usually appear in 24 hours at 37° C., and in 36 hours at 28° C. The young deep agar colony of type A strains appears as a thin translucent disc having a small dense knot or ' nucleus ' on or near the periphery at one side. The edge of the disc is either flattened or indented at the point nearest the knot. The disc is more or less completely filled with clear spots or ' vacuoles ', which are in reality gas bubbles (cf. van Ermengem's description). The outline of the colony is always clear-cut and definite. In old colonies the nucleus may develop short stubby processes. The gas bubbles, when visible, are diagnostic of *B. botulinus*, no other anaerobe forming this type of colony (G. Burke, 1919[2]). Other types and strains may form colonies, the outlines of which are less well defined and more woolly.

Spores. Spores are formed in all media under artificial cultivation, and in human foodstuffs, and in forage, &c., should the organism develop in them. Some variation occurs in different strains as to the most frequent position of the spore in the rod, and the presence of clostridial forms. With American strains sporulation takes place at all temperatures up to 37° C. ; at the last named commencing spore-formation may be observed after 24 to 48 hours' incubation.

Resistance of Botulinus Spores.

The bacillus without spores is readily killed by heat and chemical agents, but the spores are very resistant. The resistance is somewhat variable, depending partly upon the particular race, and partly upon the culture medium on which the organism has been grown. In general, the spores of type A and of type C are more resistant than those of type B.

Dozier (1924), experimenting with a number of substances, found that 10 per cent. hydrochloric acid at room temperature kills all spores within an hour, and commercial formalin diluted with an equal volume of water in 24 hours.

Many investigations have been made on the heat-resistance of botulinus spores. The results are influenced by a number of factors—age, number present in the material tested, composition and hydrogen ion concentration of the media. The young moist spores possess the highest resistance, and among a large collection of spores the majority are destroyed relatively quickly by a comparatively low temperature, but a small minority possess considerable thermal resistance. Hence, in experiments in which only a small number of spores is treated, the thermal death-point may appear to be considerably lower than when a large number of spores is tested.

Tanner and Dack (1922) found that the dry spores withstood a dry heat of 110° C. for over 2 hours, of 140° C. for 15 to 30 minutes, and of 160° and 180° C. for 5 to 15 minutes. Esty and Meyer (1922) found that the spores in 20 gm. of natural soil were killed in less than 3 hours at 100° C.

Dickson, Burke, Beck and Johnston (1925) carried out an extensive series of experiments on the thermal death-point of moist cultured spores. The spores were obtained from young cultures, and a constant number of spores (50 millions) was exposed to a constant temperature in small sealed glass tubes in a bath for a stated time, and the minimum and maximum periods of survival and of death were observed, as follows :

	100° *C.*		107° *C.*		115° *C.*		121° *C.*	
	S.	D.	S.	D.	S.	D.	S.	D.
Minimum.. ..	60′	75′	15′	20′	4′	5′	2′	3′
Maximum ..	360′	375′	80′	85′	42′	43′	22′	23′

S = survived, D = dead.

The thermal resistance of the spores in brain-broth cultures, 7 strains, and in canned beans, 1 strain, was investigated by Dickson, Burke and Ward (1919). The cultures and beans were emulsified and diluted, and then heated in tubes in a water-bath. All the strains survived 90° C. for 3 hours, 7 strains survived 95° C. for 3 hours, and 6 strains survived 100° C. for 2 hours.

Bengtson (1924) tested several strains of type C and found that they all survived boiling in a water-bath for 15 minutes, 4 survived 30 minutes, 1 survived 60 minutes, but all were destroyed after 90 minutes' boiling. Culture No. 94 heated for 15 minutes at 85° C. survived, but was destroyed in the same period at 90° C. This agrees closely with van Ermengem's results (death in half an hour at 80° C.).

After exposure to a temperature approximating to the thermal death-point, the spores may, if not actually killed, lie dormant for long periods before they germinate—up to 22 months—even in a satisfactory culture medium.

Fermentation Reactions.

Van Ermengem noted that abundant gas formation occurred in glucose broth but that no gas was produced from lactose or saccharose, and that milk was unchanged. The report on the ' Anaerobic Infections of Wounds ' of the Medical Research Council (1919) states that milk is unchanged, and that glycerol, glucose, maltose, lactose and starch are fermented, galactose, saccharose, inulin, mannitol, dulcitol, and salicin are not fermented.

According to Reddish (1921), *B. botulinus* ferments with gas production glucose, lactose, saccharose, maltose, lævulose, and galactose (only an occasional bubble with the last named). The gas formed in these fermentations is carbon dioxide.

Kendall, Day and Walker (1922) investigated the fermentative powers of six different strains and found that lactose was not attacked. All the strains fermented glucose, maltose, and glycerol, and two of the strains also fermented starch and saccharose. It will be observed that the results obtained vary, particularly as regards lactose and saccharose. With lactose, the different results observed may possibly be accounted for in part by the variable influence of the heat employed in the preparation of the culture medium, as has been noted in the case of *C. diphtheriæ*. The fermentation reactions of different strains and ' types ' doubtless vary somewhat, but Bengtson (1924) never observed fermentation of lactose and saccharose. According to her, all strains ferment glucose, lævulose, maltose, glycerol, and dextrin with acid and gas, the amount of gas, however, varying much. Raffinose, rhamnose, xylose, arabinose, inulin, dulcitol and mannitol were not fermented by any strain.

Proteolysis.

B. botulinus is a proteolytic organism and digests and darkens, but does not blacken, minced-meat and brain-broth media. Such cultures have generally a rancid, but not putrefactive, odour. All strains liquefy gelatin, though the rate of liquefaction varies, and it may be necessary with some strains to cultivate at 37° C. in order to obtain it. Loeffler's blood serum is commonly slowly liquefied, coagulated egg-white medium is sometimes digested, and fluid egg medium is sometimes coagulated and digested (coagulated egg-white medium is nutrient beef-broth with a piece of coagulated egg-white ; fluid egg medium is a solution of yolk and white in water). Milk is unaltered by some strains, others may curdle and digest it with some gas production, others may slightly acidify it without other change.

Taking the digestive action upon coagulated egg-white as a basis of differentiation, Bengtson (1924) suggests a division of *B. botulinus* into proteolytic and non-proteolytic varieties, those which digest egg-white being termed *C. parabotulinum*, and those which do not do so, *C. botulinum*. Classified in this manner, almost all type A and B strains are proteolytic, type C strains and Culture No. 94 are non-proteolytic.

SEROLOGICAL REACTIONS.

Agglutination. Agglutinating sera for *B. botulinus* were obtained by Starin and Dack (1923) by inoculating rabbits intraperitoneally with 6 or 8 doses of young toxin-free culture at intervals of 3 to 5 days. Animals receiving spores only failed to show evidence of agglutinin formation. The titre of the sera ranged from 1 : 1,000 to 1 : 2,000, and agglutination was best carried out by a macroscopic method, the mixtures being incubated at 50° to 54° C. for 2 hours. The sera of types A and B each reacted quite specifically, and the tests indicated the existence of at least four agglutination sub-groups of type A, and three sub-groups of type B. One A strain neither produced agglutinin nor agglutinated. Confirmation of these results was obtained by saturation tests, the agglutinin being absorbed by massive doses of a young culture.

Complement-fixation. The presence of complement-fixing bodies in the serum of immunized animals was also demonstrated by Starin and Dack (1924). The best antigen for use in the test was found to be a solution of toxin-free organisms derived from a young culture, disintegrated by alternately freezing and thawing about ten times. Types A and B reacted specifically to complement-fixation.

These results were confirmed by Gunnison and Schoenholz (1927[1]), who also found that the serological races already differentiated by agglutination and agglutinin-absorption tests could be distinguished by complement-fixation.

In preserved vegetables, such as peas, corn, and spinach, inoculated with *B. botulinus* and incubated at 37° C., antigenic substances are formed capable of absorbing complement in the presence of ordinary botulinus antitoxin, as well as with the antiserum produced by immunization with bacterial cells. These antigenic substances were demonstrated after incubation of the vegetables for periods of 60 hours, 8 days, and 8 weeks.

Specificity, however, was not well marked, and the two types A and B could not be distinguished. The ratio between antigenic and anti-complementary values of such vegetable antigens was also small, so that very careful titration of all the reagents was necessary in order to avoid non-specific reactions.

Precipitins. Gunnison and Schoenholz (1927[2]), obtained a precipitin reaction which was highly specific for both type A and type B, and also for the serological subgroups within the type. The sera employed were the same as had been used in their complement-fixation tests; the antigen consisted of bacterial cells disintegrated by freezing and thawing according to the method of Starin and Dack.

VARIATIONS AMONG STRAINS AND TYPES.

It is questionable whether a strain derived from van Ermengem's original Ellezelles strain now exists, but, according to Bengtson, Culture No. 94 of our National Collection of Type Cultures corresponds so closely with the original van Ermengem strain that this strain should be considered the type species. Immunologically it is a type B strain, culturally it resembles a type C strain.

The following differences observed among the various strains and types are mainly taken from Bengtson's work, which was largely based on cultures derived from single-cell isolations.

Morphology, &c. The young rods of all strains were very similar in size, form, and arrangement. *B. parabotulinus* of Seddon is stated to be larger and to have a greater tendency to chain-formation than the type.

American type A and B strains were actively motile, Culture No. 94 was motile, and *B. parabotulinus* feebly motile. In contrast to these, type C strains appeared non-motile in ordinary cultures, though in very young cultures a slight motility might be observed. The flagella of the American type A and B strains were long and numerous—15 to 30 or more. The flagella of type C strains and of Culture No. 94 were shorter and fewer—5 to 15 for the former, and 15 to 20 for the latter.

Sporulation in type C strains and in Culture No. 94 was terminal with little distension of the rod, in Type A and B strains it tended to be median or subterminal, with many clostridial forms.

Cultivation. The capacity for growth of the various strains varied considerably. Types A and B generally grew luxuriantly, type C less so, with failure to grow on certain media, and Culture No. 94 was most difficult of any to grow. Liver-digest media proved the most favourable culture medium. Van Ermengem states that the Ellezelles strain grew best at a temperature of 20 to 30° C., but this has not been the experience with the more recently isolated strains.

Characters of colonies. In glucose-agar type A strains yielded the typical compact discoidal colonies described by Burke, type B strains showed a tendency to form woolly colonies, and Culture No. 94 a somewhat fluffy colony with dense centre, while type C strains formed diffuse woolly colonies without nucleus; the colonies of *B. parabotulinus* were diffuse and not solid (the Ellezelles strain formed compact colonies).

Proteolytic action. In minced-meat medium type A and B strains darkened the meat and digested it, but Culture No. 94 and type C strains produced no change. All strains liquefied gelatin. Type A and B strains completely liquefied Loeffler's blood-serum; but Culture No. 94 and some type C strains did not liquefy, and other type C strains very slightly liquefied, this medium. Type A and B strains digested coagulated egg-white, and coagulated and digested fluid-egg medium; Culture No. 94 and type C strains had no action on these media.

Fermentation reactions. In brom-cresol milk all strains caused slight acidity. Type A and B strains in addition precipitated and digested the casein, with some gas production. Culture No. 94 and type C strains neither precipitated nor formed gas.

The common action of all strains on fermentable substances has already been detailed. In addition, type A strains alone ferment salicin, Culture No. 94 ferments inositol and adonitol, and type C strains ferment inositol and galactose.

Type C organisms usually fail to produce gas in solid culture media. Wagner (1924) found that type C strains and Seddon's *B. parabotulinus* did not grow on anaerobic blood-agar plates, as the others would.

Toxicity and serological relationships. In the chicken, which is relatively resistant to the toxin, the fatal doses of the toxins of type A, type B and type C are in the proportion of about 1 : 25 : 75. That is, the A toxin is certainly 25 times as toxic for chickens as the B and C toxins. The toxin-antitoxin relationships have already been stated. Complement-fixation and agglutination tests also distinguish the various types from one another. As regards Seddon's *B. parabotulinus,* Pfenninger (1924) found that two type C agglutinating sera failed to agglutinate it even in low dilution, though the antiserum prepared with Seddon's strain agglutinated type C strains in low dilution.

In view of the differences existing among the members of the group of organisms constituting *B. botulinus,* particularly in their toxin-antitoxin relationships, Weinberg and Ginsbourg (1926) prefer to regard the term '*botulinus*' as a generic, and not a specific, one.

PATHOGENESIS OF BACILLUS BOTULINUS.

Whether botulism may be caused by infection with the organism and its spores, or whether it results solely from ingestion of the preformed toxin, is a question the importance of which was fully realized by van Ermengem, and his answer to it is quite definite: botulism is an intoxication, produced only by ingestion of the toxin. He concluded from his experiments that the organism does not multiply in the tissues, and he remarks 'the *Bacillus botulinus* behaves in the tissues, the blood, &c., as a most harmless saprophyte, like the common hay-bacillus' (1897^2).

Van Ermengem was able to recover the organism from the spleen and digestive tract of one of his fatal cases, and this observation has been confirmed by later work, and experimentally Orr (1922) found that *B. botulinus* can be recovered from the organs of animals which have been fed or injected with toxic cultures or with toxin-free spores of the organism. Orr and also Coleman and Meyer (1922) found that experimental botulism may be produced in laboratory animals by the feeding or injection of massive doses of toxin-free spores (45 millions subcutaneously, or 90 millions *per os*). He concludes that botulism in man due to the ingestion of spores, apart from toxin, must be very rare, if it occur at all. Starin and Dack (1925) confirmed Orr's work, and doubt if the toxin-free spores play any role in human botulism. They found that the toxin-free spores of both type A and type B, if introduced in very large numbers (60 to 200 millions) intravenously, intraperitoneally, or *per os*, may form toxin in sufficient amount to induce experimental botulism, though many of the inoculated animals escape intoxication. They were able to recover the organism from the organs of the affected animals, and in those that were unaffected the spores may lie latent for long periods, though still viable. The so-called kataphylactic agents of Bulloch and Cramer (1919), such as calcium chloride and quinine, which render toxin-free tetanus spores capable of growth and toxigenesis in the tissues, do not exert any influence on the pathogenicity of botulinus spores.

The toxin-free spores of *B. botulinus* inoculated in moderate doses up to a few millions produce no noticeable effect; subcutaneously, there is no local reaction. The organism appears to have no capacity for infecting, either generally or locally, and it was never recovered from war wounds.

Reputed Manifestations of Botulism in Animals.

Evidence has been adduced, apparently conclusive in some instances but less so in others, that several diseases of animals are manifestations of botulism. Bulbar paralysis and paralysis of the locomotor system are the cardinal symptoms in all these diseases.

Forage poisoning from the consumption of hay and ensilage occurs in horses, mules and cattle in all parts of the world, including this country, and is frequently known as ' cerebro-spinal meningitis '. In an outbreak of this disease at Ottawa, Illinois, among horses, Graham and Brueckner (1919) isolated a bacillus from the fodder (corn ensilage) which was identical with *B. botulinus*, a type B organism according to G. Burke (1919[1]). Its toxin fed to a mule produced a condition similar to the natural forage poisoning. G. Burke (1919[1]) also isolated *B. botulinus* from mouldy hay, the cause of forage poisoning among horses and mules at Oakdale, California, but corroborative feeding experiments were not made in this instance. In an outbreak of fodder poisoning among cattle, *B. botulinus*, type B, was isolated by Graham and Schwarze (1921[2]). Cattle injected with type B botulinus antitoxin were fed with the incriminated fodder (corn ensilage), and showed no signs of illness, but as the control animal also remained unaffected the experiment is inconclusive.

In an outbreak of fodder poisoning in New South Wales in 1924, causing the deaths of three horses and five cows, Seddon (1926) demonstrated the presence of *B. botulinus*, type B, in the maize ensilage, the cause of outbreak.

Grass sickness is an important disease attacking horses on pasture in some districts in Scotland. Tocher and co-workers (1923) isolated *B. botulinus*, type A, in a reputed outbreak of this disease. The toxin of the organism was neutralized by American type A antitoxin, the blood of recovered animals was stated to contain antitoxin protective against type

A toxin, and animals inoculated with a botulinus toxin-antitoxin mixture were stated to be protected to a considerable degree against the disease. Recently, however, some doubt has been cast on this work. It is difficult to understand how botulism can be contracted by pasture-fed animals, in which grass sickness almost exclusively occurs: in the rare instances in which the disease attacks stable-fed animals, some fresh grass has always been given them. Nairn (1928) states that in true grass sickness there is neither paralysis of the locomotor system nor paralysis of the tongue, both of which accompany botulism. Pool (1928) mentions seven named diseases of the horse, all of which have symptoms very similar to one another, and to those usually associated with grass sickness. It seems not unlikely that 'grass sickness' is an ill-defined disease and includes more than one affection, and that possibly one of them is botulism.

Limberneck, a disease of poultry (so-called from the weak, pendulant condition of the neck), has been identified as a form of botulism. Graham and Schwarze (1921[1]) isolated type A in an outbreak from the crop of affected birds and from their food. Type C may also be a cause of this disease, but not type B. The intoxication is derived from corn fodder, restaurant garbage, and the like.

In a paralytic disease of cattle occurring in Victoria, Australia, Seddon (1922) isolated from a bone of a dead animal a toxicogenic bacillus closely resembling, but not identical with, *B. botulinus*, type C, to which he gave the name *B. parabotulinus*.

Lamsiekte, a disease of cattle occurring in South Africa and often occasioning considerable loss, has been investigated by Theiler and co-workers (1927), who have shown that it is due to the ingestion of botulinus toxin. The cattle gnaw the bones and offal of animals that have died on the veld, and if these are infected with an organism of the *B. parabotulinus* type, and toxin has been produced, they become poisoned, and lamsiekte is the result. The reason for the perverted appetite of the cattle is of considerable interest. The soil and vegetation of the veld in many localities are very deficient in phosphorus. In consequence, the animals suffer from phosphorus deficiency, and to supply the want they eat carcass debris, which they would otherwise shun. It had previously been shown that a ration of bone meal or other phosphorus-containing compounds markedly reduced the incidence of lamsiekte, because the animals have then no need to feed on offal. Animals other than cattle are sometimes attacked, such as poultry, the ostrich and goat. The organism seems to be identical with Seddon's *B. parabotulinus*, but varieties may exist, e.g. *B. parabotulinus bovis* and *B. parabotulinus equi*.

Botulinus Toxin.

B. botulinus is an organism which produces a potent exotoxin, and the conditions under which this is formed have been the subject of much research.

Formation of the toxin. Van Ermengem obtained the most potent toxin in a double strength nutrient broth containing 2 per cent. of glucose, and Hall and Peterson (1923) found that the non-fatal and fatal subcutaneous doses for the guinea-pig were respectively 0·1 c.cm. and 1·0 c.cm. of a plain broth toxin and 0·0001 c.cm. and 0·002 c.cm. of a glucose broth toxin, the cultures in each instance being incubated for 11 days. There are indications, however, that some American strains produce stronger toxins in the absence of glucose. The inhibitory action of glucose, when it occurs, is due to the acid which is formed. It has been found that glucose tends to favour toxin-production with type A strains, and to inhibit it with type B and C strains (Wagner, Meyer and Dozier, 1925).

Many media, including some of a purely vegetable nature, yield a powerful toxin with appropriate cultivation, but the most potent toxins are obtained by the use of minced-meat broth and beef-heart peptic-digest liver broth. The optimum pH value lies between 7·4 and 8·0, and toxin is formed at temperatures from 20 to 37° C. The optimum temperature of cultivation for toxin production is usually about 35° C., the maximum amount of toxin being present on about the eleventh day of cultivation. At the lower temperatures toxin production is slower, though may ultimately reach the same degree, but below 20° C. no toxin is formed even after five months' cultivation. Toxin production is progressive and in a suitable culture medium may first be demonstrated after 24 to 48 hours incubation at 35° C., when the subcutaneous m.l.d. for the guinea-pig will probably be about 1·0 c.cm. of the filtered broth. The toxicity increases, and on the eleventh day or thereabouts the m.l.d. may be 0·00001 c.cm. or less, after which toxicity tends to decrease. In a peptone medium, the content of peptone may, without influencing growth and toxin production, vary from 2 or 3 to 25 per cent., but more than 30 per cent. inhibits growth and toxin formation. Sodium chloride, similarly, up to 5 per cent. is without influence, but 8 per cent. is inhibitory (Thom, Edmondson and Giltner, 1919).

Different strains of *B. botulinus* vary in their capacity to form toxin, and cultures may lose their toxigenic power. Bengtson made single-cell isolations from a type C culture, which was toxic and derived from a well-isolated single colony. Of 3 single cells isolated, 1 was toxic, the 2 others were non-toxic. Further single-cell isolations made from the toxic and non-toxic strains yielded toxic and non-toxic substrains from the toxic strain and non-toxic substrains only from the non-toxic strain. Seventeen additional single-cell isolations from the toxic substrain yielded 15 toxic cultures and 2 non-toxic cultures.

Stability of toxin. The fluid toxin exposed to light and air undergoes rapid deterioration, but if kept in sealed tubes in the dark deterioration is much less rapid. Deterioration is more rapid the higher the temperature at which the toxin is kept. A dried or precipitated toxin is much more stable, even if exposed to the air and light.

Sommer *et al.* (1926) have obtained a 25 to 50-fold concentration of toxin by adsorbing a toxin broth with aluminium hydroxide, washing out the toxin from the aluminium hydroxide with ammonium hydrogen phosphate solution, dialysing the wash, and evaporating it at 40° C.

Effect of physical and chemical agents on the toxin. Heat is destructive to the toxin, but for a rapid effect the temperature of exposure must be not less than 75 to 80° C. The toxins of different strains vary somewhat in their heat resistance. Orr (1921), testing the toxins of 10 A and B strains, found that they were all destroyed within from 30 seconds to 5 minutes at 80° C., from 2 to 18 minutes at 72° C., and from 10 to 85 minutes at 65° C. Bengtson, with a type C toxin, found that it still retained some toxicity after heating to 75° C. for 30 minutes, and to 80° C. for 15 minutes, but exposure to 80° C. for 30 minutes completely destroyed it.

Light is destructive to the toxin but not rapidly so, exposure to direct sunlight for 40 hours being necessary to ensure complete destruction. The dried toxin is more resistant to heat and light than the fluid toxin ; there is little deterioration at 37° C. in one month, and in diffuse daylight in three months (Schoenholz and Meyer, 1924).

Van Ermengem states that amyl alcohol, ether, and benzol in acid or slightly alkaline media dissolve scarcely appreciable amounts of toxin, and contact with them does not injure it. The toxin is precipitated by tannin, lead acetate, zinc chloride, and neutral salts without loss of activity, but is destroyed by the chlorides of gold, platinum, and mercury. Oxidizing agents, such as potassium permanganate and chloride of lime, destroy it, but the gases CO_2, H_2S and O_2 have little action.

Ethyl alcohol is destructive of the toxin. Armstrong, Story and Scott (1919) observed that if alcohol were added to it so as to form a mixture containing about 32 per cent. of alcohol and allowed to stand for a few minutes, subcutaneous or intraperitoneal inoculation of guinea-pigs with amounts equivalent to 20 minimal lethal doses failed to kill, and Bronfenbrenner and Schlesinger (1921^2) found that alcohol to the extent of 20 to 30 per cent. in the mixture was capable of destroying the toxin, the destructive action being completed within 5 to 10 minutes at 37° C.

Iodine and formalin transform botulinus toxin into an anatoxin (pp. 388, 395).

Botulinus toxin is unaffected by peptic digestion and by short periods of tryptic digestion. Thus, a tryptic digest containing 0·5 per cent. of sodium carbonate, incubated for 4 hours at 38° C., did not weaken it (Schübel, 1923). But alkalis, if the action is at all prolonged, destroy its toxicity. Van Ermengem found that sodium carbonate in the proportion of 0·5, 1·0 and 3·0 per cent. rendered the toxin innocuous to rabbits after 24 to 48 hours' incubation, and a mixture of toxin and water was rendered instantly inert when mixed with an equal volume of 3 per cent. sodium carbonate solution, and Schübel states that the toxin is destroyed by caustic soda, 0·8 per cent., after 18 hours' incubation.

Dilute acids, on the other hand, are without action. Van Ermengem found that tartaric and lactic acids in the proportion of from 1 to 3 per cent., and hydrochloric acid in the proportion of from 0·5 to 1 per cent., did not lower the toxicity in 24 to 36 hours at 35° C. Bronfenbrenner and Schlesinger (1924) found that botulinus toxin resists a degree of acidity equivalent to that of the stomach, even when exposed thereto for 24 hours at 37° C. They also demonstrated (1922) that acidity to a degree of pH 4·0 actually increases the activity of the toxin to the extent of 30 to 100, or more, fold, the minimal lethal dose diminishing from 1/300 c.cm. to 1/10,000 c.cm. or less. But no larger amount of antitoxin is required to neutralize the more potent acidified toxin than the original toxin ; i.e. the combining power of the toxin does not increase with the increase of toxicity.

Other toxic substances. In addition to the specific toxin, other toxic substances seem to be present in the toxin broth. Thus, when an animal is injected with a large dose of toxin, 0·5 c.cm. or more, it frequently dies within a few minutes. If the toxin be precipitated with alcohol, this toxic substance remains in solution ; it is also dialysable and is markedly thermo-stable. The substance is probably some by-product of the bacterial metabolism (Bronfenbrenner, Schlesinger and Orr, 1921). The first two authors (1921[1]) have also found that a botulinus toxin purified by precipitation with ammonium sulphate retains its full potency by injection, but is one hundred times less toxic by the mouth. By addition of the substance removed by the precipitation to the purified toxin, the original oral toxicity is restored. Further information respecting these other toxic substances is desirable.

Origin of the toxin. Little information exists as to the mode of formation of botulinus toxin. It may be a natural excretory product of the bacterial metabolism. The fact that growth and toxin production do not necessarily run parallel is somewhat against this hypothesis. Wagner, Meyer and Dozier favour the ' enzyme hypothesis ', formulated by Dernby and Walbum for diphtheria toxin. This supposes that the bacilli undergo autolysis and liberate enzymes which split certain proteins of the culture medium and of the organisms to lower degradation products, and some of these intermediate split-products constitute the toxin. Some facts tend to support this hypothesis in the case of botulinus toxin. Thus an incubated mixture of botulinus toxin with heterologous botulinus antitoxin, e.g. type A toxin with type B antitoxin, is more toxic than the toxin alone, and a mixture of botulinus toxin with normal guinea-pig leucocytes incubated for several hours has its potency much increased. On the other hand, botulinus toxin differs fundamentally in some respects from diphtheria toxin, for the toxin once formed is fairly resistant to the action of proteolytic ferments, such as pepsin and trypsin, and the autogenous ferments of *B. botulinus* have no effect on its toxin. Some work by Nelson (1927) suggests that botulinus toxin is elaborated within the bacterial cells in intimate association with the bacterial globulin. The toxin may be liberated from the cells by autolysis, and the amount of toxin set free is proportionate to the amount of globulin which appears in solution. The toxin, however, is not identical with the globulin, for the latter may be removed by peptic digestion, leaving the toxin. The mode of derivation of botulinus toxin deserves further study.

Other products. Various enzymes are formed in the culture medium digesting meat and egg albumin, liquefying gelatin, curdling and digesting casein, &c. These are ' mobilized ' as required ; for example, gelatinase is not formed in a spinach infusion medium. Fleming and Neill (1927) found that a type A strain formed maltase and lipase, the former being endocellular, the latter extracellular. Lactic, butyric, valeric and acetic acids are formed.

Hæmolysin is formed in most media, its formation being favoured by the presence of 1 per cent. of glucose or 20 per cent. of heated sheep serum. It appears within 16 to 18 hours, or less if growth is very active, and disappears within 48 hours. The botulinus hæmolysin is very sensitive to the action of heat, light and mechanical agitation. It is destroyed by heating to 60° C. for 10 minutes, and disappears slowly at room temperature (Schoenholz, 1925). On a blood-agar plate a clear zone of hæmolysis appears around each colony after 3 or 4 days' incubation at 35° C. Type A is less hæmolytic than type B.

Flocculation occurs when botulinus toxin and antitoxin are mixed *in vitro* and incubated, but is not very marked, nor very exact quantitatively (Bronfenbrenner and Reichert, 1925).

Anatoxin. Weinberg and Goy (1924 and 1925) observed that the toxicity of botulinus toxin is destroyed by treatment with formaldehyde and with iodine. The toxin is converted by formaldehyde into an anatoxin. Weinberg and Goy experimented with quantities of formaldehyde varying from 1 part per 1,000 to 6 parts per 100, incubating the mixtures at 37° C. and testing them regularly at intervals of 4 to 5 days. With 8 parts of formaldehyde per 1,000 parts of toxin, the toxin becomes inactive and comparatively non-toxic at the end of from 12 to 17 days. Rabbits treated with this mixture became immunized and yielded an antitoxic serum.

Weinberg and Goy also applied the method for the immunization of a horse, an animal which is very sensitive to botulinus toxin and not easy to immunize with it.

Immunization by the mouth. Tchitchkine (1905) found that rabbits given increasing doses of botulinus toxin by the mouth could tolerate 4 to 6 times the minimal lethal dose *per os* for an untreated animal. But such immunized animals were only feebly, if at all, protected against subcutaneous inoculation of the toxin. Lippmann later (1910), by feeding mice with increasing doses of the toxin, obtained a protection against four minimal lethal doses *by the mouth,* but such protected animals succumbed to one minimal lethal dose injected *subcutaneously.*

Weinberg and Goy (1925), giving animals varying doses of anatoxin by the mouth, found that they would resist small *subcutaneous* doses of toxin—up to 4 m.l.d.'s.

Cutaneous reaction. Botulinus toxin provokes no reaction on the skin, so that a cutaneous reaction cannot be carried out (de Lavergne, 1924).

NEUTRALIZATION OF TOXIN BY NON-SPECIFIC SUBSTANCES.

An old observation of Kempner and Schepilewsky of the protective action of olive oil against botulinus toxin was confirmed by V. Burke, Elder and Pischel (1921), who tested a number of substances to determine whether they would be useful for the non-specific treatment of botulism. The method employed was to make a mixture of the substance with a known amount of filtered toxin (2 to 8 m.l.d.'s) and inject into guinea-pigs, or to feed rabbits with the toxin and then to administer the substance, immediately or with an interval, after the toxin. Several cubic centimetres of olive oil emulsified with the toxin prevented death from 3 but not from 4, minimal lethal doses. The death of rabbits fed with toxin could be delayed by feeding with olive oil immediately and at later intervals, and somewhat similar results were obtained with a liquid paraffin (' petrolatum '). Other substances tested—butter, milk, egg-white and yolk, sugar, brain and gelatin—had little or no effect. Iodine to which starch had been added to prevent tissue necrosis, afforded some protection when injected with the toxin, but not when injected separately. Gram's iodine solution also afforded some protection when fed to rabbits immediately after the toxin. Vinegar fed to a rabbit after the toxin also seemed to afford some protection, and alcohol acts similarly. Larson and Eder (1926) found that sodium oleoricinate, which destroys the toxicity of diphtheria toxin, is without action on botulinus toxin.

EFFECT OF TOXIN ON THE BODY.

Most animals, including man, poultry and some other birds, such as the ostrich, are susceptible to botulinus toxin, both by inoculation and in some instances by ingestion, though the lethal dose may vary widely in different species and according to the mode of administration.

Van Ermengem studied at some length the action of the toxin of the Ellezelles strain, which he found to be more active than that of the Darmstadt, and still more so than that of the Iseghem, strains. Rabbits, guinea-pigs and mice were most susceptible. By subcutaneous injection, the lethal dose for rabbits was 0·00005 to 0·0001 c.cm., fatal in 18 to 36 hours. Death ensues with symptoms of general or local motor paralysis. The head may incline to one side, the forelegs be extended, and the hind legs be contracted on the abdomen, or the hind legs may be paralysed so that the animal creeps painfully on its forelegs. Other symptoms that may occur are salivation, mydriasis, flaccidity of the third eyelid, exophthalmos, and anorexia, with retention of urine or fæces. The symptoms come on suddenly, usually a few hours before death, so that when life is prolonged there may be an incubation period of 3 or 4 days. Death may be preceded by clonic convulsions and squealing. Guinea-pigs were particularly susceptible, a dose of 0·00005 to 0·0001 c.cm. being fatal in 18 to 36 hours. There may be a total paralysis of all muscles, so that the animal lies motionless with outstretched limbs and the whole body appears flat. With smaller doses, various paralyses may occur, as of the hind legs. An early and characteristic symptom is loss of resistance of the abdominal muscles, quickly followed by salivation and aphonia. If the injection be made into a limb a condition of local paralysis may first supervene, analogous to that which sometimes occurs with tetanus toxin.

Mice were somewhat variable in their susceptibility, about one-third of the guinea-pig lethal dose being commonly fatal.

For the rhesus monkey a dose of 0·001 to 0·01 c.cm. caused death in 24 to 36 hours, with dilated pupils, bilateral ptosis, discharge from mouth and nose, cough and dyspnœa. In cats, 0·1 to 1·0 c.cm. killed in 6 to 8 days with marked mydriasis and immovable pupil, complete paresis of the third lid, with fixed eyeball, aphonia, prolapse of the tongue, mucous discharge from the mouth, croupy cough, retention, and general muscular weakness—a most characteristic picture. Larger doses after a latent period of a few hours, were followed by a general paralysis and death with collapse within 16 to 24 hours. Smaller doses, e.g. 0·01 c.cm., may be followed by a cachectic condition which may last some weeks. White rats were but slightly susceptible, and dogs and hens withstood enormous doses, a dog surviving after as much as 30 c.cm. A dose of 0·1 to 0·5 c.cm. injected into the breast muscles killed pigeons in a few days with more or less complete paralysis of the wing muscles, ptosis, and regurgitation of greenish fluid. Horses and goats were very susceptible, 0·03 c.cm. being a fatal dose for a goat. Frogs and fish were completely refractory.

Ingestion. It is almost a universal rule that the true toxins derived from cultures of pathogenic organisms, as well as snake venom, are harmless, even in large doses, when administered by the mouth ; to exert their effect they must be introduced parenterally. The failure of a toxin to act when given by the mouth is generally ascribed to destruction by the digestive juices. Botulinus toxin is a notable exception and is active whether administered *per os* or parenterally, and as previously mentioned is not destroyed in the presence of acid, nor by peptic or tryptic digestion. Some animals which are susceptible to inoculation of botulinus toxin are, however, resistant to ingestion, and the active dose by ingestion is larger, often enormously so, than that by inoculation.

Van Ermengem found that monkeys, guinea-pigs and mice were susceptible to small doses by ingestion, a drop or two of toxin given on a piece of bread killing in 24 to 36 hours. Rabbits were far more resistant, 5 to 10 c.cm. being required to kill by ingestion. Cats also were resistant and often withstood enormous doses, though sometimes, after a large dose, they died after a lapse of some days with paralysis and marasmus. The dog, rat, and hen were similarly resistant to very large doses by ingestion.

Dack and Gibbard (1926) found hogs to be very resistant to oral administration of type B toxin, and to withstand 10 million mouse inoculation m.l.d's.

Some of the more recent American results are somewhat different from those of van Ermengem, probably because a toxin derived from a different strain of organism was used (the van Ermengem strain more nearly resembles the American type B and C strains, as previously mentioned). Thus Dickson found chickens susceptible both to subcutaneous injection and to feeding, and dogs to subcutaneous injection with 1·0 c.cm. of a type A toxin. Graham and Eriksen (1922) determined the fatal doses for the dog of an unfiltered type A toxin to be 0·1 c.cm. by injection, and 100 c.cm. by ingestion if food be withheld. A type B toxin failed to induce symptoms by either method: even 80 c.cm. subcutaneously, and 15 c.cm. intravenously, were almost without effect. Bengtson found that a type A toxin is much more toxic for chickens than either type B or type C toxins. The fatal doses in inoculation tests were roughly 2,000 guinea-pig m.l.d's. for type A toxin, 10,000 for type B, and 25,000 for type C. By feeding, the fatal dose of type A toxin was about 20,000 guinea-pig m.l.d's., while type B and C toxins produced severe symptoms, but not death, in doses of from 100,000 to 250,000 guinea-pig m.l.d's.

With lamsiekte toxin (*B. parabotulinus* type), Theiler and his collaborators found dogs, rats and swine completely resistant, guinea-pigs, rabbits and mice being highly susceptible (to inoculation).

The horse, ass, sheep, goat, ox, chicken, turkey, duck and ostrich are all susceptible to ingestion of one or other type of botulinus toxin under natural conditions, so that the diseases known as forage-poisoning, grass sickness (?), bulbar paralysis of cattle, lamsiekte, and limberneck of poultry arise. The insusceptibility to botulinus toxin of the rat and pig, which are omnivorous feeders, and of the dog and cat and probably other carnivors, by ingestion, is of interest and in accord with some of their habits.

Lamsiekte toxin is exceedingly toxic for cattle, much less so (one-fiftieth) for horses; Seddon's *parabotulinus* toxin is more toxic for horses than for cattle; type A toxin is not very toxic for cattle; type B toxin is somewhat toxic for horses.

The variable susceptibility of different animals to oral administration of botulinus toxin may be due to a variable degree of destruction or adsorption of the toxin by the intestinal contents, or to differences in permeability of the intestinal wall to the toxin. No evidence was obtained by Dack and Gibbard (1925–6) of adsorption by the intestinal contents of the guinea-pig *in vitro*. In blood perfused through a loop of rabbit's intestine into which toxin had been injected, very small amounts of toxin were present, 0·5 c.cm. producing botulism in a mouse, but not 0·1 c.cm.

In similar experiments performed on two hogs, 0·5 c.cm. of the perfused blood of one animal taken 1½ hours after injection killed a mouse, but in the blood of the second animal no toxin was detected. Toxin was not found in 1·0 c.cm. of blood of a hog fed 1½ hours previously with 9,000,000 m.l.d's. of toxin for the mouse. Two rabbits given large doses of toxin into the small intestine developed botulism 7 days later, but no toxin was found in 0·5 c.cm. of blood taken 90 minutes and 18 hours after the dose. In mice fed with botulinus toxin but not developing symptoms, toxin was present in the stomach and intestine 3 hours after the feed, but not 12 hours later. These permeability experiments appear, therefore, to be inconclusive and somewhat contradictory.

Intraperitoneal injections of similar doses of toxin produce a more rapid effect than subcutaneous ones. Dyspnœic symptoms are generally prominent, appear early, and may follow a very small dose. The same applies to intrapleural injections.

Mice inoculated intraperitoneally with botulinus toxin show such characteristic symptoms that Bengtson (1921) has suggested their use in testing for the presence of the toxin. Dyspnœa is prominent, the respirations falling rapidly from 120–160 to 20–30 per minute. Respiration becomes costal with strong contraction at each breath, and death ensues within 3 or 4 hours.

Intracranial and *intracerebral inoculation* are not followed by earlier, or more marked, symptoms than subcutaneous or intraperitoneal injections.

Intraocular inoculation is not followed by ocular symptoms any sooner than subcutaneous inoculation. Ophthalmoplegia and mydriasis appear in *both* eyes simultaneously and at the proper time after inoculation into *one* eye.

Intravenous injections are particularly fatal to goats and horses, a fact of some importance in connection with antitoxin production. In other animals much the same acute symptoms are produced as follow large subcutaneous injections.

PATHOLOGICAL LESIONS CAUSED BY BOTULINUS TOXIN.

Botulinus toxin produces comparatively insignificant lesions, macroscopic and microscopic, in the organs and tissues. Van Ermengem described the lesions in the Ellezelles cases as consisting of a hyperæmic state, more or less pronounced, in all the organs, often accompanied by vascular ruptures and sanguineous extravasations ; these are the most constant findings both in man and in animals. If the disease is protracted, necrotic changes and fatty degeneration may occur in the liver, spleen and kidneys, and if the toxin has been ingested a hyperæmic condition with hæmorrhagic foci may be observed in the digestive tract.

In the brain, cord and cranial and spinal nerves there are no very obvious lesions beyond vascular engorgement and minute hæmorrhages. The older observations indicated that the cellular elements of the central nervous system are affected, namely those of the grey substance of the anterior horns of the cord and of the nuclei of the medulla and pons. The lesions of the nerve cells were described as consisting of a break-up, ending in chromatolysis and destruction of the Nissl bodies. Cowdry and Nicholson (1924), however, from an examination of a large series of sections from a number of animals and comparison with similar sections derived from normal animals were unable to detect any alteration in the Nissl bodies or in the nerve fibres. Beyond occasional meningitis and vascular engorgement with slight thrombosis and infiltration, very little was to be found in the brains of experimental animals. Wilbur and Ophüls (1914) in a human case similarly found the Nissl bodies to be normal in size and arrangement and to stain well, especially those of the large motor ganglion cells. They observed macroscopically a moderate hyperæmia of the cerebral vessels, especially at the base of the brain and in the region of the fourth ventricle, where there was also some œdema. A condition not previously recorded was the presence of multiple thromboses in many of the larger and smaller arteries and veins, the thrombi being rich in polymorphonuclear leucocytes. The vessels chiefly affected were the basilar artery and its pial branches, the vertebral artery, many of the pial veins at the base of the medulla and pons, and the veins of the tela choroidea and posterior part of the third ventricle. Small perivascular hæmorrhages were often present about the thrombosed vessels. Thrombi were not observed elsewhere than in the brain.

Dickson (1918) confirmed the presence of thrombi in experimental animals. Thrombus formation commences during the first 24 or 48 hours of intoxication, and reaches full development soon afterwards, and is more frequent in rabbits than in guinea-pigs. The thrombi consist of dense masses of fibrin, which is arranged in thick bands, and have many polymorphonuclear leucocytes enmeshed between the strands; they are frequently adherent to the sides of the blood vessels. Visible conglutinated blood platelets are usually absent, but occasionally hyaline masses are present which are probably composed of platelets. The prethrombic stage mentioned consists of agglomerations of fibrin or the presence of hyaline masses. At times the red-blood corpuscles have disappeared from the lumen of the vessels, but in many instances they are intimately mingled with the leucocytes between the strands of fibrin. The thrombi are most frequent in the meninges and tissues of the central nervous system, but may be found in every organ if searched for. They are found both in arteries and in veins, but much more in the latter, and in the organs are more frequent in the smaller vascular branches which ramify in the parenchyma. Cowdry and Nicholson (1924), however, doubt if thrombosis is more than of occasional occurrence; it may be that the stage of intoxication and the species of animal employed materially influence the findings.

The site of action of the toxin has been the subject of much discussion. The toxin does not appear to attack the nerve cells and ganglia of the central nervous system. According to Edmunds and Long and Keiper

(1923 and 1924) the essential action of the toxin is upon the motor end plates of skeletal muscle and of the diaphragm, with paralysis of the vagus and parasympathetic nerve endings. This view accords with the absence of changes in the nerve cells and fibres (Cowdry and Nicholson, 1924), and with the fact that guinea-pig brain does not absorb or neutralize botulinus toxin *in vitro* (Colman, 1924).

Botulism in Man.

It has been previously shown (p. 382) that botulism can hardly arise from the ingestion of botulinus spores, and that the disease in man (and in animals also) develops only as a result of the ingestion of the preformed toxin with some article of food.

Susceptibility of man. That man is very susceptible to botulinus toxin by ingestion may be inferred from the results of animal experiments, and is proved by the small amount of a food containing toxin which is sufficient to cause botulism. For instance, in the remarkable outbreak of botulism at Canton, Ohio, in 1919, caused by eating canned ripe olives, one of the victims died after eating only half an olive; and Dickson records two fatal cases from eating two teaspoonfuls of preserved corn and from 'nibbling' a pod of home-canned string beans respectively; in a third case the patient was very ill but recovered, after 'tasting' a pod of home-preserved string beans. The American cases have resulted from the toxins of types A and B, but not apparently from that of type C, which, however, seems to be of much rarer occurrence than the two others. If the Ellezelles and some of the other European strains belonged to the type C group, all three types of *B. botulinus* must be regarded as capable of inducing botulism in man. The mortality may be as high as 100 per cent., or as low as 8 to 9 per cent., but this low mortality is the exception, and the average for all cases is probably 60 to 70 per cent.

Symptomatology and course. The first symptoms usually develop in from 18 to 24 hours, but may occur earlier or may be delayed for 2 or 3 days or even longer. The earliest symptom is commonly an indefinite feeling of fatigue, sometimes with headache or dizziness and definite muscular weakness. Acute gastro-intestinal disturbance is usually absent, but occasionally early nausea and vomiting may occur, and constipation is the rule. Disturbances of vision come on early, with diplopia, paralysis of the third nerve, ptosis, mydriasis, loss of the light reflex, and weakness and paralysis of accommodation. Difficulty in swallowing and in articulation follow, with dryness of the throat and sluggishness of the tongue. The pulse may be slow or rapid, the heart's action weak, and disturbance of respiration may become severe. Death usually occurs from cardiac or respiratory failure, generally in from 4 to 8 days after the onset. The temperature is generally sub-normal, unless broncho-pneumonia supervenes, true paralysis of the skeletal muscles is seldom found, and sensory disturbances are almost completely absent.

The *post-mortem appearances* are insignificant and ill-defined. The only constant finding is marked congestion of the central nervous system and of the thoracic and abdominal viscera. Microscopic examination shows the presence everywhere of marked congestion with minute hæmorrhages. Thromboses of the type already described (p. 393) may occur in the meninges and tissues of the central nervous system and in the organs (Semerak, 1921), but are probably neither so constant in, nor so characteristic of, botulism as Dickson thought.

In the few cases of human botulism in which it has been looked for, antitoxin has not been found to be present in the blood. Thus, de Lavergne (1924) tested the serum of a botulism patient on the fifth, eighth, fifteenth and thirtieth days after the commencement of the attack and was unable to find that it possessed any trace of neutralizing power against the toxin. In the case of horses that had recovered from a disease they regarded as grass sickness, Tocher *et al.* (1923) claimed to have found botulinus antitoxin in their blood.

Botulinus Antitoxin.

Antitoxins active against the respective toxins of all the types and varieties of *Bacillus botulinus* may be produced by inoculating animals with increasing doses of the corresponding toxin. Goats and horses are to be preferred for this purpose, as rabbits and guinea-pigs are most difficult to immunize. These antitoxins are strictly specific for each type of organism, but by treating an animal with the toxins of the different types a polyvalent serum may be obtained.

Kempner (1897) was the first to prepare a botulinus antitoxin, in the goat. The value of the serum so prepared was such that 1·0 c.cm. contained 100,000 units, the unit being equivalent to a test dose of toxin which killed a guinea-pig weighing 250 gm. in 2 days.

The interaction between botulinus toxin and antitoxin, as with diphtheria and tetanus antitoxins, is a binding, and not a destructive one. In the earlier stages of interaction dissociation of the toxin-antitoxin complex may be brought about by various means, such as dilution, addition of acid, introduction into the stomach, &c. Thus, in a test by Bengtson with type A toxin and antitoxin, it was found that 0·1 c.cm. toxin mixed with 0·5 c.cm. antitoxin and afterwards fed to a guinea-pig caused the death of the animal, though with 0·5 c.cm. toxin and 0·5 c.cm. antitoxin injected subcutaneously, the animal survived. In the first case, the acidity of the gastric juice presumably dissociated the toxin-antitoxin complex, and as the toxin is absorbed more rapidly than the antitoxin, death ensued.

Production of Antitoxin on the Large Scale.

By R. A. O'Brien.

Toxin of *B. botulinus* has been produced on the large scale in order to immunize horses or mules for the production of antitoxin. With toxin-antitoxin mixtures or formalinized toxin (i.e. 'toxoid' or 'anatoxin';

Weinberg and Goy, 1924, p. 148), animals can be readily immunized, and there seems little doubt that if it were considered advisable to immunize human beings by the subcutaneous injection of these materials, satisfactory protection would be achieved.

In order to make toxin for the immunization of horses, a strain of the bacillus is grown in ordinary peptone nutrient broth with a deep layer of autoclaved meat at the bottom, the final pH being 8·0. Bengtson (1924, p. 86) suggests 'a pH of 8·0 to 8·5 for the protèolytic and pH 7·0 to 7·5 for the non-proteolytic, strains'.

In serological laboratories the broth is usually kept at about 35° C.; reasonably good toxin is produced in 7 to 11 days. Weinberg and Ginsbourg (1927) mention a growth period of 2 to 4 weeks. The culture is filtered through Berkefeld or Chamberland candles and the filtrate may contain toxin of which from 0·0001 c.cm. to 0·000001 c.cm. injected subcutaneously may kill a guinea-pig or mouse within a few days. The toxin is usually moderately stable, but may deteriorate; it is, therefore, wiser to precipitate it by saturation with ammonium sulphate. In the dry state it is stable for long periods. The immunization of horses may be commenced by the subcutaneous injection of approximately neutral toxin-antitoxin mixtures, or with toxoid made by the addition of iodine or formalin to toxin. In some series, very small doses of toxin have been used initially; Wadsworth (1927) refers to the use of 'low grade' toxin with a m.l.d. of 0·005 c.cm., but these methods are risky and one can increase the doses only very slowly. Wadsworth thinks that 100 times the m.l.d. for the guinea-pig by the subcutaneous method is fatal to a horse, and records the loss of 2 horses out of 3 in an attempt to immunize rapidly. With some horses it is possible to use toxin initially and to increase the doses rapidly; this suggests that they possess natural immunity. An average course of immunization would be represented by increases of from 30 to 50 per cent. in the successive doses, and injections once or twice a week. Trial bleedings of the horses are tested from time to time, and at the end of from 3 to 6 months antitoxin of reasonable titre should be present. The serum is titrated in the usual way by the subcutaneous injection of mixtures of antitoxin with the test dose of toxin into the guinea-pig or mouse.

Apparently the only standards at present issued are those of the United States Hygienic Laboratory, the unit quantity of standard antitoxin for types A and B being originally based on 10 times the amount of antitoxin protecting against 100 m.l.d's. of a certain toxin in use. The quantity of any toxin just failing to neutralize one-tenth of this unit of antitoxin is the test dose, and may vary with different toxins of different age and constitution from 50 to 100 m.l.d's. In testing, the guinea-pig subcutaneous method is used, and the neutralizing dose of antitoxin is taken as that quantity which keeps the injected guinea-pig alive for 4 days. 'Serums testing as high as 200 units per cubic centimetre are considered satisfactory antitoxins' (Bengtson, 1924, p. 88). The specificity of A and B toxins and antitoxins is high. It is possible by immunizing horses with the three toxins to produce trivalent antitoxin. Since A and B are the types most frequently met with, the more usual course is to immunize horses separately against A and B toxins and to mix the two sera. Antitoxin C can be made separately ; it is rarely called for. R. A. O'B.

Treatment of Botulism with Antitoxin.

The only remedy likely to be of the slightest value in the treatment of botulism is botulinus antitoxic serum. Even this is unlikely to save life if the disease has progressed to a late stage. It is, therefore, of paramount importance to give the antitoxin as soon as the earliest symptoms, such as blurred or double vision, ptosis, giddiness, difficulty in speech or swallowing, &c., suggest a diagnosis of botulism. As in tetanus, the antitoxin is unlikely to be of use when the symptoms of poisoning are well established.

The botulinus serum should be a polyvalent one ; at least for the types A and B. The method of administration by preference should be the intravenous one, and large doses only can be expected to be effectual. The intravenous dose should be at least 20 c.cm., and better 50 c.cm., of the antitoxin, previously warmed to the temperature of the body and injected very slowly. If for any reason the intravenous route is impracticable, the intramuscular method should be used. Intrathecal inoculation of antitoxin is not likely to be of service, if, as seems probable, the lesions are peripheral and not central, but it may be worthy of trial. If so, in addition to the intravenous (or intramuscular) injection, an intrathecal injection of 20 to 30 c.cm. of the warmed serum might be given at the same time, after withdrawal of a similar amount of cerebrospinal fluid. Additional daily intravenous or intramuscular injections of the antitoxin should be given for several days if symptoms continue.

The usual precautions must be taken before injection of the serum to avoid the risk of serious symptoms developing in a person sensitized to horse serum, all therapeutic serums being derived from the horse.

Bronfenbrenner and Weiss (1924) observed experimentally that anæsthesia delays the progress of the intoxication, even after it has commenced, and morphine has the same power and may render antitoxin more effective. Full doses of morphine may, therefore, be a useful adjunct to antitoxin treatment, as well as allaying some of the symptoms most distressing to the patient.

A prophylactic dose of 10 c.cm. of antitoxin intramuscularly should be given to any person who may have consumed some of the suspected food, but has not yet developed symptoms, to be followed by the full doses intravenously should symptoms supervene.

Occurrence of Bacillus botulinus under Natural Conditions.

Bacillus botulinus is widespread in nature. It occurs in the soil and on fruit and vegetables. Van Ermengem made repeated efforts to isolate the organism from natural products but never found it except in two specimens of ham which had caused outbreaks of botulism. He made cultures from garden soil, street dirt, mud from ponds and rivers, horse and cow manure, and duck and fish excreta. Kempner (1897) recovered a strain from the intestinal contents of a normal hog, and this remained the solitary instance of recovery of *B. botulinus* from material other than that causing botulism until 1918. The presence of *B. botulinus* in the intestine of the normal hog is exceptional, for Dickson (quoted by Burke, 1919[1]) examined 250 animals without recovering it. In 1918, Burke (1919[1]) made a survey in five districts in central California and 235 cultures were prepared from an extensive series of materials, including tap-water, hay, leaves, vegetables and fruits in various conditions, insects and snails, garden soil, manure, and the contents of the digestive tract of birds. The outcome was that seven cultures containing botulinus toxin were detected, and in four other cultures evidence of the presence of a weak botulinus toxin was found. Both type A and type B toxins were identified, and the materials containing *B. botulinus* included cherries, bush beans, spiders, mouldy hay, and manure from a hog which had recovered from botulism three months previously.

Some of the inferences from this work are: (*a*) *B. botulinus* is not necessarily associated with active decay, it may be present in the blemishes on the skin of otherwise sound fruit and vegetables; (*b*) *B. botulinus* may remain in the intestinal tract of an animal for a long period after contaminated food has been eaten; and (*c*) there is evidence to suggest that *B. botulinus* may be associated with, or be disseminated by, spiders or other insects.

Meyer and Dubovsky (1922[1]) examined 624 specimens of soil, vegetables, fruits, farm feeding-stuffs, manure and sewage collected in thirty-six counties of California, and isolated toxic cultures of *B. botulinus* in approximately 30 per cent. of the samples. More than 50 per cent. of samples of virgin soil yielded evidence of the presence of *B. botulinus*, type A predominating. In cultivated garden and field soils type B was the predominant form. These authors consider that the evidence suggests that the natural habitat of *B. botulinus* is virgin mountain or forest soil. Coleman (1922), on the other hand, found that cultivated soils of a narrow belt on the coast of California were heavily contaminated with spores of type A.

Meyer and Dubovsky (1922[2]) also demonstrated the presence of type B in the soil and in vegetable specimens collected in Belgium, Denmark, England, the Netherlands and Switzerland. Type A was consistently absent in Europe, type B alone being found. They suggest that Gaffky's Darmstadt strain was a type A, and remark that if so, it is probably the only type A strain hitherto isolated in Europe. Type A was, however, the cause of the Loch Maree outbreak, and was also isolated by Tocher *et al.* in their work on equine grass sickness, both in Scotland. In England, *B. botulinus* was widely distributed, being found at or near Eastbourne and Brighton; Ealing; Shottle Hall, Derby; Breinton, Hereford; and Shotley Bridge, Durham. It is of interest that type B is the predominant form in the Eastern Atlantic Coast States, type A being more frequent in the Western Pacific Coast States of the United States; type B is also the more frequent form in China and Hawaii (Schoenholz and Meyer, 1922).

After its discovery in 1924, type C was also found to be an inhabitant of the soil. Damon and Payabal (1926) examined 62 samples of soil collected in Maryland and found that 8 samples contained Type A alone, 25 type B alone, and 5 type C alone. Types A and C were present together in 4, and types B and C in 5, samples; all three types were not found together.

The presence of *B. botulinus* in human fæces appears to be exceptional, although the spores must not infrequently be ingested. Thus, Easton and Meyer (1924) examined the stools of 88 healthy individuals who were known to be ingesting spores on raw fruits and vegetables, without finding the organism. From the fæces of a case of botulism, Wheeler and Humphreys (1924) isolated type B on the sixth day of the disease. Easton and Meyer (1924) also examined 50 samples of animal excreta and isolated type A from three specimens of hog fæces and from two specimens of cattle fæces.

Tanner and Dack (1922) examined 73 samples of soil and found *B. botulinus*, type B, present in 11 of the samples. In 6 samples of fresh animal excreta they found the organism to be present in 3 samples of hog fæces, but absent from samples of fæces of 3 dairy cows. Tanner and Dack also found type B present in 2 out of 10 samples of human fæces derived from apparently healthy individuals.

Hall and Peterson (1924) consider Dubovsky and Meyer's results for the prevalence of *B. botulinus* in cultivated soil too low, and found that of 20 samples of arable soil in California, 70 per cent. were contaminated with the organism, 50 per cent. with type A and 15 per cent. with type B. They employed a minced-meat broth with 1 per cent. of glucose.

Development of Bacillus botulinus in Foodstuffs.

The fact that botulism occurs from the *ingestion* of the botulinus toxin, and not from *infection* with the organism, at least under natural conditions, is undoubted, and it is of importance, therefore, to gain an understanding of the conditions governing the development of *B. botulinus* in foodstuffs, with toxin-formation.

The outbreaks of botulism associated with animal products—ham, sausage, fish, and the like, are easy to understand, for a meat medium is an excellent pabulum for the growth of *B. botulinus* and the development of its toxin, given a sufficient lapse of time before consumption and a temperature not below 20° C. The frequent association of botulism with the consumption of ham is explained by the fact that ham may be kept for a considerable time before consumption and is commonly eaten uncooked. Butcher's meat is eaten fresh and is cooked before consumption and, therefore, never gives rise to botulism. Game which is frequently kept until it is ' high ' might be expected to cause botulism occasionally, but the thorough cooking to which it is generally subjected before consumption may be the safeguard.

B. botulinus will, however, develop and form its toxin equally well in a purely vegetable medium, as was first shown experimentally by Dickson (1915). In fact, botulism in the United States has been associated with vegetable products much more frequently than with animal ones. Again, fresh vegetables, fruits and salads, cooked or uncooked, do not give rise to botulism, because, although they may be contaminated with the spores of the organism, there has not been time for the development of the toxin. With preserved and canned products the necessary time element will generally exist, and in the case of canned products the anaerobic condition and the comparative absence of non-sporing saprophytes tend to promote the development of *B. botulinus* if present.

Schoenholz, Esty and Meyer (1923) made a systematic study of 346 cans of commercially prepared foods artificially inoculated with washed and heated spores of *B. botulinus* (to remove adherent toxin). After inoculation, the cans were kept some at room temperature, some at 35° C., and some alternately at both these temperatures, for periods varying from 10 days to 12 months, and were then examined. They found that under these conditions corn, peas, sweet potatoes, pumpkin and salmon undergo spoilage regularly and quickly, and the decomposition products are very toxic. Spoilage and toxin production were irregular in asparagus and beet, and growth at room temperature was frequently retarded. Even when toxin production occurs it is not always accompanied by an amount of spoilage sufficient to warn the consumer. Multiplication of *B. botulinus* in spinach and stringed beans is irregular and not always accompanied by evolution of gas, and toxic spinach may be normal in appearance. Spoilage with toxin formation occurs in evaporated milk, but is irregular ; the organism does not seem to grow in condensed milk.

Toxin production by *B. botulinus* occurs only exceptionally and after prolonged incubation in acid fruits and vegetables, e.g. apricots, cherries, peaches, plums, raspberries, strawberries, tomatoes, and sauerkraut. Thompson and Tanner (1925), employing similar methods, confirmed these results in all essentials. They found that canned apples, blackberries, gooseberries and dill pickles, all of which belong to the acid group of fruits, showed no toxin formation, while beans, hominy and shrimp became regularly highly toxic after a short period of incubation, and showed spoilage. A product like spinach, the reaction of which is normally pH 5·0, which is a preventive against the development of *B. botulinus*, becomes suitable for development if the acidity be neutralized. The reaction is greatly influenced by the quality of the material canned—a small addition of rotted spinach added to good will soon reduce the acidity to the danger point.

Starin (1926[2]) found that temperatures of 20° C. and 25° C. are equally favourable for the growth of *B. botulinus* and the production of its toxin in such vegetables as corn, stringed beans, peas and spinach. Development is more rapid at 25° C., though the toxicity eventually reaches the same level at both temperatures. There was no development of toxin at 10 to 12° C. even after five months, though the organism still remains viable and can be recovered at the end of the period. He also observed (1926[1]) that a material containing few spores is much more readily sterilized by heat than if it contain many spores. Thus with material containing only 9 to 12 toxin-free spores heated at 100° C. for 10 minutes, subcultures were not obtained in a series of 384 attempts.

Prevention of Botulism.

The occurrence of botulism is intimately associated with the eating of foods that have been kept for a time, and are not as a rule cooked, before consumption. Botulism thus arises from the consumption of ham, preserved sausages (but not of the fresh variety), preserved or canned fish, canned meats, and preserved and canned fruits and vegetables. While it may be possible to limit the consumption of such articles of diet and to substitute for them fresh foods so far as possible, their total avoidance is obviously impossible. It is particularly with home-preserved, potted, and canned, articles of diet that the risk is greatest, for the methods employed by the great canning firms are far more perfect than those employed in the home. Thorough cooking of these preserved foods whenever possible, by destruction of the toxin, would eliminate most, if not all, of the risk of botulism, but cannot be applied to all preserved products.

The Research Committee of the National Canners Association (U.S.A.) (quoted by Leighton, 1923) made some important suggestions for the prevention of botulism associated with the consumption of canned foods. They pointed out that two important safeguards consist in the careful removal of all dirt from foodstuffs that are to be preserved, together with the use of fresh and sound raw materials, treated and packed with the least delay. They further suggested that some system should be adopted whereby any can may be identified with the batch in which it was sterilized; that the date of its production be stamped upon the label, and that samples should be taken from every batch, incubated, and examined before distribution of the batch. In this manner, if it were found that a particular batch had been imperfectly treated or sterilized, this batch could be withdrawn.

For spinach and olives, which have particularly been associated with outbreaks of botulism in the United States, the State of California has made regulations defining the size of cans to be used, the amount of the contents, and the time and temperature of sterilization.

All canned vegetables and fruits should, so far as possible, be boiled after removal from the container and before consumption. Efficient sterilization is the essential safeguard from botulism in the case of commercially preserved foods.

The action of heat on botulinus toxin in canned foods has been investigated by Tanner and Twohey (1926). Spores of a type B strain, chosen as yielding a particularly resistant toxin, were used; 1·0 c.cm. of an emulsion of toxin-free spores (approximately 1,215,000 spores) was introduced into each of several tins of canned foods (meats, fish, vegetables), the holes in the cans were sealed with solder, and the cans were then incubated at 37° C. for four days or more. The cans bulged and on opening showed a decomposed appearance and had a pronounced odour. A portion of the contents was removed, distributed in narrow tubes, and heated at different temperatures for varying periods, after which guinea-pigs were fed with the material. It was found that in order to destroy the toxin, heating at 100° C. required 4 to 20 minutes; at 90° C., 25 to 40 minutes; at 80° C., 25 to 60 minutes; at 70° C., 45 to 50 minutes; and at 60° C., more than 4½ hours. In an open pan, as used in the kitchen, heating after the boiling point was reached for 10 to 15 minutes detoxicated some samples, but others were still toxic after 20 minutes.

The more acid the raw material, the less the danger, and fruits, tomatoes and rhubarb having a pH value of 4·5 or under are unlikely to be subject to botulinus spoilage. Where the pH value is higher, sterilization should be all the more carefully performed.

The toxin once formed in a foodstuff, may persist for long periods. Starin (1927) found that the toxin of both type A and type B formed in preserved or canned corn, peas and salmon, will withstand exposure to drying, light, and seasonal variations of temperature and persist for at least 90 days. Van Ermengem found that putrid macerations of toxic ham still retained their toxicity, and Starin states that the putrefaction of toxic vegetables and meats does not cause the disappearance of the toxin.

The addition of cane sugar to beef broth up to a concentration of 64 per cent. does not prevent the development of *B. botulinus*, nor the formation of toxin, and certain fruits canned in sugar syrup form suitable soils for the development of *B. botulinus*, e.g. peaches, apricots and pears (Dickson, Burke and Ward, 1919). A lemon juice brine containing 2½ oz. of salt, 3½ oz. of sugar and 7 oz. of lemon juice per gallon does not prevent the development of *B. botulinus*, nor the formation of its toxin.

A feature of considerable importance is that botulinus-infected foodstuffs in which toxin has developed do not necessarily show signs of spoilage. In addition to the direct contamination of foodstuffs with soil or dirt containing botulinus spores, the experiments of Burke suggest that indirect contamination is a possibility through the intermediary of spiders and insects, and many years ago Bail (1900) demonstrated that several species of flies are capable of acting as porters of *B. botulinus* and its spores.

For the prevention of botulism among animals, control and selection of their food may be of service in some instances, e.g. the exclusion of spoiled or fermented fodder or of old domestic and restaurant refuse. In the case of lamsiekte, if the animals be supplied with a ration of phosphorus-containing food, such as bone meal, they will no longer seek the toxin-containing offal, and will not, therefore, become poisoned.

In reputed grass sickness attacking horses in Scotland, Tocher and his collaborators employed a toxin-antitoxin mixture for immunization. Of 961 inoculated animals 27 died of grass sickness; of 355 uninoculated animals serving as controls 33 died, a percentage mortality of 2·81 and 9·30 respectively, a difference of 6·5 per cent., which is statistically significant.

An anatoxin, prepared from botulinus toxin by the action of formaldehyde (see pp. 388, 395), would probably serve as an efficient prophylactic vaccine without risk to the animals.

Diagnosis of Botulism.

The symptoms of the disease in man are so characteristic that there should be no difficulty in diagnosis, particularly as more than one person will generally be attacked at about the same time. Laboratory methods are not available, even if life is prolonged, and a cutaneous reaction gives no result. If death ensues, there usually is nothing characteristic or specific about the post-mortem appearances. The peculiar thromboses which have been described might be looked for, and if found would be significant. An attempt might be made to isolate *B. botulinus* from the spleen, but a negative result would carry no weight.

Botulism in animals similarly will be recognized only if and when its possible occurrence is appreciated, and the characters of the intoxication are known to the observer.

If a particular article of diet or a foodstuff be incriminated upon clinical and epidemiological data, the effect of feeding, or the inoculation of an extract, may be tested upon animals. Ten c.cm. of the vegetable juice or pickling fluid, e.g. brine, may be filtered through paper and then centrifuged, or, if necessary, filtered through a filter candle. A portion of the centrifuged liquid, or of the filtrate, is boiled for 30 minutes. Two mice are inoculated intraperitoneally with 0·5 c.cm. of the unheated and heated material respectively, or guinea-pigs similarly with 1·0 to 2·0 c.cm. If botulinus toxin be present in sufficient amount, the animals injected with the unheated fluid will succumb in from 4 to 72 hours, while those receiving the heated fluid will survive. Animals non-immunized and immunized with antitoxin, if this is available (but it should be a polyvalent serum), may also be fed with the material. With a foodstuff containing little fluid, a portion may be fed to animals, or some of the material may be ground up, extracted with saline, and the extract, filtered if necessary, inoculated into, or fed to, animals. Mice inoculated with botulinus toxin show such characteristic symptoms (p. 392) that Bengtson (1921) recommends their use for the purpose. The type of toxin present can be determined by neutralization tests with type antitoxins.

ISOLATION OF *B. BOTULINUS* FROM CONTAMINATED MATERIAL.

For the isolation of *B. botulinus* from material containing it, the van Ermengem broth, or a minced-meat broth, may be employed and may succeed if the spores are numerous, but not unless. The same holds good for direct cultivation in agar media. Dubovsky and Meyer (1922) after many trials elaborated the following method, in which the medium employed is the beef-heart peptic-digest liver broth of Stickel and Meyer (1918). The procedure is as follows : From 20 to 50 gm. of soil, vegetable, fruit, meat or other material are placed in a 250 c.cm. flask and sufficient sterile saline solution (pH = 7·0) is added thoroughly to suspend the material, which should be ground or chopped fine. Very acid materials should be first neutralized. The suspension is then heated to 60 to 70° C. for 1 to 2 hours, or to 80° C. for 1 hour. After this, and while still warm, 100 c.cm. of the special medium are added, the mixture is covered with a layer of liquid paraffin, or may be exhausted of air through a piece of tubing passed through a rubber cork in the flask, the tube being sealed off after the exhaustion is completed. The cultures are then incubated at 35° C. for 10 days. At the end of this time, a small sample of the supernatant fluid is withdrawn by means of a sterile pipette, and is centrifuged at high speed for one hour, and 2·0 c.cm. of the upper clear fluid are injected subcutaneously in the median abdominal line of a guinea-pig weighing 250 to 300 gm.; two or three animals should be inoculated. The animals are observed from day to day, and any one that dies is examined. The post-mortem findings consist of a slight infiltration or œdema, with or without hæmorrhage, at the site of inoculation, or frequently no observable change, and congestion of the viscera with dark-coloured liver and spleen.

Centrifugates which are fatal to guinea-pigs in 2 c.cm. doses within 4 days may be further identified by antitoxin-neutralization tests, with type A, B and C sera, but those producing the first symptoms in 3 or 4 days with death on the fifth to the eighth day are to be regarded as ‘ weak ’ toxins and are unsuitable for the further identification test.

Only those cultures which yield filtrates of which a dose of 2·0 c.cm. kills the guinea-pig within 18 hours are suitable for isolation of *B. botulinus* in a pure state. Should the guinea-pigs succumb to another infection, as may happen when a centrifugate, and not a filtrate, is used for inoculation, some of the enrichment culture is filtered through a filter-candle, and the filtrate is then tested in a similar manner. When many cultures are being dealt with, centrifuging instead of filtering saves time and labour.

For isolation of *Bacillus botulinus* from the enrichment culture, after incubation of the broth cultures at 35° C. for 10 days, a portion of the sediment—1 to 2 c.cm.—is sealed in a small glass tube which is then heated to 80° C. for 1 hour, or is boiled in a water-bath for 10 minutes. Small amounts of this heated material are then immediately plated in blood-agar plates, or sown into deep shake cultures of agar, glucose-agar, or, best of all, Heller's (1921) liver-agar medium. After incubation of these cultures, suspicious colonies are picked out, subcultured, and examined in detail.

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CHAPTER XII. BACTERIAL FOOD POISONING.

BY WILLIAM G. SAVAGE.

ACUTE poisoning from the ingestion of food may be non-bacterial in origin. The poisonous substance may be simple inorganic bodies such as zinc or lead salts, more complex bodies such as belladonna or solanin, or highly complicated toxins, non-bacterial in origin, such as are found in poisonous mushrooms. The acute illnesses in sensitized individuals which follow the use of certain perfectly sound foods, and which are now grouped as food allergy, may also be included under food poisoning. The great majority of cases are, however, caused by the toxic products of bacteria. From the bacteriological standpoint they are conveniently grouped according to the bacteria concerned. These bacteria are *B. botulinus*, Salmonella bacilli, dysentery bacilli and indeterminate groups of bacilli.

Botulism.

This very definite type of food poisoning is due to *B. botulinus*. The different types of this bacillus and the bacteriological features of botulism are described in Chapter XI. The only points discussed here are those relating to the vehicles of infection and the changes produced in foods.

Vehicles of infection. The common vehicle on the Continent of Europe is some form of meat, especially sausage or ham. The following table compiled by Bitter (1921) of comparatively recent outbreaks illustrates this point:

Fish	1
Sausages of different kinds	16
Ham, horseflesh	18
Meats, apart from ham or horseflesh	9
Home-preserved beans	1
Other food materials	13
	58

In U.S.A. canned and bottled fruits and vegetables have been the common vehicle. In California and other endemic areas the spores have been found on market fruits and vegetables (Burke, 1919; Meyer and Dubovsky, 1922). Geiger, Dickson and Meyer (1922) collected 91 reports of outbreaks in that country since 1899. In 82 the food responsible was ascertained, and is shown in the following table compiled from their report. Only 30 outbreaks were proved bacteriologically. The outbreaks affected 345 people, with 213 deaths, a case-mortality rate of 61·7 per cent.

Food vehicle	Home preserved	Commercially preserved	Total
String beans	17	3	20
Corn	9	1	10
Asparagus	5	0	5
Apricots: Pears	5	0	5
Spinach	2	6	8
Beets	1	2	3
Olives	0	10	10
Other fruits or vegetables	1	1	2
Ham: Pork	3	1	4
Beef products	3	0	3
Sausages	1	2	3
Fish: Shellfish	1	3	4
Other foods	3	2	5
	51	31	82

Physical changes in the food. The growth of *B. botulinus* in foods is usually associated with obtrusive signs of physical deterioration, i.e. gas development, a sour odour and a slightly altered appearance, due to the fact that the bacillus is a gas-producing proteolytic organism. A number of outbreaks have occurred, however, in which no signs of spoilage are recorded. Schoenholz, Esty and Meyer (1923) tabulate 15 such outbreaks, 5 being caused by spinach. In the only British outbreak, at Loch Maree, alterations were not noticed in the vehicle, which was wild-duck paste, but they were probably present and overlooked, since the isolated organism inoculated into a sound jar of this paste caused definite physical changes (Savage and White, 1925^2). Experimental inoculation of sound tins of food with *B. botulinus* has verified the rare occurrence of toxin production without spoilage changes (Schoenholz, Esty and Meyer, 1923; Thompson and Tanner, 1925; and Starin, 1926). Such cases occur with acid foods, and the probable explanation is that in the early stages sufficient toxin to make the food poisonous may be produced before spoilage changes result, and then the acid produced is sufficient, added to the natural high acidity, to inhibit further growth and the development of definite spoilage.

Toxicity of ingested spores. There is no authentic instance recorded in which botulism has developed in man unless botulinus toxin was taken into the alimentary canal. This fact confirms van Ermengem's conclusion that the organism itself is non-pathogenic and is only poisonous when the vegetative form can grow and produce toxins. Edmondson, Giltner and Thom (1920), in an experimental investigation, were able to cause the death of guinea-pigs by the subcutaneous injection of enormous numbers (30 million of detoxified spores). Very much larger doses (78 to 192 million spores) were required to produce botulism when given by the mouth. This possibility has been confirmed by Orr and Coleman and Meyer (1922) and others. The number of spores ingested by man from soil-contaminated fruit or vegetables is likely to be so few that their development into vegetative forms with toxin production is a negligible danger, although the above results admit its theoretical possibility.

Heat-resistance of the spores. This is of paramount importance in connection with outbreaks with canned foods as the vehicle. Numerous investigations show that there are great variations in resistance, partly conditioned by the particular strain of *B. botulinus* used, the number of spores present, and particularly the hydrogen ion concentration of the medium. Esty and Meyer (1922) found the heat resistance of 109 strains from various sources varied from 3 to 80 minutes at 105° C. in a phosphate solution pH 7·00 to 7·12. The heat resistance in the juices of 17 varieties of canned food showed a variation from less than 10 minutes to 230 minutes at 100° C. Weiss (1921) found a gradual decrease in thermal resistance as the spores age, spores a month old being three times as resistant as those five months old. Esty (1923) concluded from his investigations that, assuming the most resistant spores known, the following time-temperature relationship would destroy them : 360 min. (100° C.) ; 120 min. (105° C.) ; 36 min. (110° C.) ; 12 min. (115° C.) ; 4 min. (120° C.).

Salmonella (and Dysentery) Food Poisoning.

Until recently food poisoning was not associated with bacilli of the dysentery group, but Savage and White (1925^2) found 4 out of 100 consecutive outbreaks to be due to members of this group. The symptoms and epidemiological features were in almost all the cases definitely of the Salmonella type and not dysenteric. In one outbreak *B. dysenteriæ* (Flexner, W.) was isolated and in the other three, strains culturably identical with, but differing serologically from, the Sonne-Thjøtta type.

As set out in Volume IV, the Salmonella group includes many types. Many have become specialized in their functions and habitats, and only some are associated with food poisoning. The present state of knowledge is that the majority of outbreaks are due to *B. aertrycke* and *B. enteritidis*, that a much smaller number are associated with *B. suipestifer*, Derby type, Stanley type and Newport type, while very rare food-poisoning types are type L (White, 1926) and Thompson type (Scott, 1926). Other types, such as *B. abortus equi*, *B. paratyphosus* A, and probably *B. paratyphosus* B, are not found as causes of acute food poisoning.

Three important characteristics of the Salmonella group in relation to food poisoning are their very rapid rate of growth on meat and other suitable foods (Savage, 1910; Koser, 1922; Damon and Leiter, 1927), the fact that they cause no obtrusive changes in the appearance, taste or smell of foods, and the production of toxins with considerable heat resistance. While the bacilli have no greater resistance than ordinary non-sporing bacilli the toxins withstand temperatures of 100° C. and higher, and so are not destroyed by ordinary cooking, unless very prolonged.

This difference of resistance determines two distinct varieties of Salmonella food poisoning. One, due to undestroyed toxins without living bacilli, is characterized by a short incubation period (usually 2 to 4 hours), acute and often very severe symptoms in the alimentary tract, rapid subsidence of these (often in about 24 hours) with complete recovery, except in very rare instances. When living bacilli are present with no great amount of preformed toxins the incubation period is longer, the symptoms less severe in onset but usually more prolonged, while a fatal result may occur, due to general infection.

The food-poisoning Salmonella strains are the cause of many animal diseases. For example *B. enteritidis* has been found causing disease of cows, calves and rats, *B. aertrycke* of mice, guinea-pigs and other rodents, parrots and other birds, calves, and occasionally pigs. *B. suipestifer* is a common secondary invader in pigs suffering from Hog Cholera, while at times it acts as a primary disease producer in pigs. The distribution of the other types has not yet been adequately worked out.

The available facts suggest that Salmonella strains can be divided into three classes as regards disease causation. One class comprises the strains which are found only in man, including *B. paratyphosus* A and B (and probably C). They behave like *B. typhosus* and cause disease of paratyphoid fever type but not food poisoning. The second class includes *B. pullorum*, *B. sanguinarium* and *B. abortus equi*, which originate disease in their selected animals but not in man. The third class includes *B. enteritidis*, *B. aertrycke*, *B. suipestifer* and the rarer food-poisoning strains, which are capable of producing disease in both man and animals. In man they cause illness of the food-poisoning type, never paratyphoid fever.

With so complex a group it is hardly to be anticipated that the distinctions between the three classes will invariably be completely clear-cut, but the classification suggested is probably a near approximation and corresponds accurately with the existing facts. Such a grouping assists materially in the elucidation of the source of infection in food poisoning.

The evidence strongly negatives the view that members of the Salmonella group are natural inhabitants of either the human or animal intestine. The food-poisoning types (unlike *B. paratyphosus* B) do not persist for long periods in the human intestine after acute attacks, thus causing chronic carriers. Infection of food from a human carrier as the source of the bacilli in an outbreak is extremely rare, and at the most has only been demonstrated in two or three instances.

In a considerable number of outbreaks the infected food has been from an animal suffering from disease due to a Salmonella group bacillus. For example, in Great Britain several milk-spread outbreaks have been due to a cow suffering from a *B. enteritidis* infection. In general, however, outbreaks associated with the flesh of a diseased animal are in a decided minority in this country. They are much commoner on the Continent, and are often associated with the use of the flesh of an emergency slaughtered animal.

Investigation usually shows that the food was quite sound in origin, but if an animal food, must have been infected after slaughter. Often the actual path of infection cannot be elucidated. The hypothesis advanced by Savage (1913, 1920) that infection is derived directly or indirectly from animals which either at the time are suffering from disease due to Salmonella group bacilli or are acting as temporary carriers of these bacilli fits in with the present state of our knowledge, but the subject is still obscure. Indirect infection from rats or mice would be included in this hypothesis.

Food Poisoning due to other Types of Bacilli.

In spite of much investigation the causation of a proportion of outbreaks is obscure. There is no evidence that putrefactive food as such is a cause of food poisoning and the ordinary putrefactive bacilli may be exonerated, although some temporary malaise may be set up by the consumption of such food. The evidence that organisms like *B. coli*, *B. proteus* or Morgan's bacillus can cause food poisoning is slender, and although many outbreaks have been attributed to them, critical investigation does not substantiate the association.

On the other hand, all investigators with considerable experience of food-poisoning outbreaks come across cases for which no bacterial cause can be found, and it is probable that further types of food-poisoning bacilli will be brought to light as causing a few outbreaks. Bacilli of the dysentery type have only been added since 1924.

Relative Prevalence and Different Vehicles.

A general idea of the relative prevalence of the different types of causation is obtainable from the following particulars of 100 consecutive outbreaks in Great Britain studied by Savage and White (1925^{2}).

Outbreaks probably not true food poisoning	3
Outbreaks due to members of the Salmonella group	66
,, ,, ,, ,, Dysentery ,,	4
,, ,, *B. botulinus*	1
,, of definite chemical origin	2
Cheese-poisoning outbreaks	8
Mild evanescent outbreaks	9
Outbreaks of undetected bacterial origin	7
	100

Excluding the 3 outbreaks probably not food poisoning, and including 1 cheese outbreak as certainly of Salmonella origin, this gives 67 out of 97, or 70 per cent., as due to Salmonella bacilli.

The nature of the vehicle is shown in the following table of 203 British outbreaks:

Nature of Food	*No. of Outbreaks*	*Percentage*
Canned meat	31	30·5
Canned marine products	27	
Canned fruit	4	
Milk	14	6·9
Milk products	16	7·9
Made-up meat	54	26·6
Manipulated meat	10	4·9
Fresh meat	33	16·3
Fruit and vegetables (not canned)	8	3·9
Other foods	6	2·9

The Bacteriological Investigation of Outbreaks.

The methods for the isolation of *B. botulinus* or members of the Salmonella or Dysentery groups are set out in the sections dealing with them. Only a few features more particularly related to the kind of material to be examined need be discussed here.

(a) *Materials to examine.* Any portions of the supposed peccant food; particular care is necessary to select food which is really part of that supplied to the patients. The post-mortem materials from fatal cases most useful are spleen, liver, pieces of small intestine, piece of large intestine, kidney. The stomach (ligatured) with unopened contents is sometimes valuable, especially for negative evidence. Samples of excreta and vomit, especially in the acute stage. Samples of blood from sufferers taken 7 to 10 days after the onset.

(b) *Clinical and epidemiological details.* It is essential to have these at the outset even in the incomplete state then available. They are most helpful in suggesting both lines of investigation and the further material required for examination.

(c) *Investigation of outbreaks suggesting Salmonella toxins.* This is the type of outbreak outlined above and is the one commonly found associated with the consumption of canned foods. It is usually impossible to isolate any bacilli ætiologically associated with the outbreak. Only very rarely can positive agglutination results be obtained with sera of patients. The difficulties of proof are discussed by Savage and White (1925[2]), and they describe two methods which occasionally are successful. These are the demonstration of the production of specific agglutinins in animals through the injection of emulsions of the incriminated food, and the demonstration in young rabbits of an inflammatory action on the stomach some hours after feeding with large amounts of emulsions of the food.

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